

UNIVERSITY OF ALBERTA

Genome-wide hypermutation underlies adaptive mutation

by

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fulfillment of the requirements for the degree of Master of Science

in

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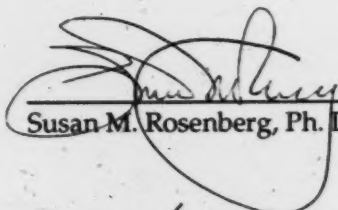
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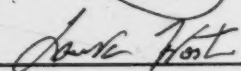
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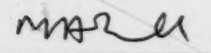
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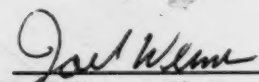
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Abstract

The RecABC-dependent "adaptive" reversion of an F-borne +1 *lac* frameshift allele in *Lac*⁻ *E. coli* has been detected in the F' and not in one *E. coli* chromosomal location tested. The purpose of this thesis is to address three questions regarding the specificity of stationary-phase mutation in this system. First, is the adaptive mutation mechanism directed to the gene whose function was selected, or do unselected loci mutate? Second, is the F' the exclusive site of Rec-dependent mutation. Third, the postulate that adaptive reversion occurs in a differentiated subpopulation is tested. Using a replica-plating screen, I find significantly higher rates of mutation associated with stationary-phase *Lac*⁺ reversion than in *lac*⁻ control populations, that either were or were not exposed to selection. The associated mutations occur in unselected genes, in all replicons in the cell, and have a similar sequence spectrum to the adaptive reversions. In addition, the *rec*-genotype of cells plated with the frame-shift bearing cells has no effect on *Lac*⁺ mutation rates. These results support models in which, adaptive mutations (i) are not directed, in a Lamarkian manner, to the selected gene, (ii) are not an F'-specific event, and (iii) occur in a differentiated subpopulation of cells exposed to selection.

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Chapter 1

Introduction

In recent years an old debate has reared its head in the scientific community. At the heart of the debate are Charles Darwin and Jean Baptiste Lamarck. Current opinion on the formation of mutation favors the Darwinian theory which contends that mutations arise randomly, prior to, and independently of exposure to a selective environment. On the other side of the debate is the Lamarckian view that organisms direct mutation in response to exposure to a selective agent or environment. That is, an organism can distinguish between mutations that are improvements and those that are not. An article by John Cairns and colleagues that appeared in *Nature* in 1988, suggested that bacteria could in fact direct mutation to a locus under selection. This appeared as a resurgence of the discredited views of Lamarck, and challenged the established view of Darwinism.

This introduction will review the classic experiments that cemented Darwinian theory in the minds of scientists as the only route to mutational variation. It will also discuss experiments that provide evidence for the existence of a mutational process that allows organisms to direct mutation apparently in response to a selection imposed by the environment. The body of this thesis is concerned mainly with this underlying question: can a bacterial cell direct mutation to a locus under selection while leaving the rest of the genome unaffected?

Evidence for the existence of random spontaneous mutation in bacteria

Earlier this century, there was much discussion, especially as it relates to bacteria, about whether mutation might be "directed" or "post-adaptive" in the sense that the appearance of mutations were a direct result of the environmental conditions. The alternative, and now widely accepted view, is that mutations arise spontaneously, without regard to utility, and if beneficial the environment simply serves to select for the maintenance of the mutation. In 1943 solid evidence for the occurrence of random spontaneous mutation in bacteria was provided (Luria and Delbrück, 1943). Salvador Luria and Max Delbrück made use of a fluctuation analysis to show that mutations in *Escherichia coli* arise prior to the bacteria being exposed to the selection. Luria and Delbrück exposed sensitive *E. coli* cells to bacteriophage T1 and selected for those cells that had mutated to resistance to the phage, such that they could grow and form a colony. The reasoning behind the fluctuation test is as follows. If a mutant arises by chance during the growth of a culture, prior to exposure to T1 bacteriophage, it will give rise to a clone of mutant progeny. It follows then, that if the first mutant arises early in the growth of the culture it will give rise to a large number of mutant progeny. If the first mutant arises late in the growth of the culture, it will give rise to only a few mutant progeny. Therefore, among parallel cultures grown from identical inocula, the distribution of the numbers of mutants will be characterized by a large variance. However, if the mutations arise only after exposure to the selective agent (i.e.: adaptively), then every cell will have an equal probability of mutating, such that the numbers of mutants arising from parallel cultures will have the narrow variance of the Poisson distribution. Luria and Delbrück plated parallel cultures of *E. coli* B on nutrient agar plates seeded

with bacteriophage T1. They observed a high variance distribution and considered their results as,

"proof that... the resistance to virus is due to a heritable change of the bacterial cell which occurs independently of the action of the virus."

Newcombe (1949) noted that Luria and Delbrück's fluctuation test had gained only limited recognition, possibly due to the statistical and indirect nature of the argument. Consequently, Newcombe (1949) developed a more direct experimental approach. He plated *E. coli* cells, which are susceptible to phage T1, on agar and incubated the plates to allow some growth. On alternate plates he then spread a saline solution over the surface of the agar to redistribute the bacteria. All plates were then sprayed with phage T1 and the resistant mutants (which form colonies) were counted subsequently. If mutation to phage T1 resistance is induced by selection (i.e.: exposure to T1), one would expect that all the cells after the period of incubation, prior to T1 exposure, would be sensitive to T1 and spreading would only re-distribute sensitive bacteria. Therefore, no differences between the numbers of resistant bacteria would be observed for spread and unspread plates. On the other hand, if mutation to phage resistance occurs prior to exposure to the selection, then both resistant and sensitive bacteria would be present after the initial growth period. As a result, spreading would redistribute a cluster of resistant cells (a clone) and one would observe many more resistant colonies on the spread plates than on the unspread plates. Newcombe observed as much as a 50-fold increase in resistant colonies on the spread plates, providing direct evidence for the idea that mutations arise before exposure to selection.

In 1952, the Lederbergs developed a technique called replica-plating which allowed for the indirect selection of cells from a non-selective medium (Lederberg and Lederberg, 1952). The technique involves placing velveteens on a block of wood, such that one could make imprints of colonies from non-selective plates and these imprints could then be transferred to other selective plates. This technique identifies siblings of cells that were pre-existing on a non-selective plate and exposes the siblings to a selection. By allowing mutant isolation without exposing the original colony to the selective condition, the Lederbergs demonstrated the pre-existence of mutations to both phage T1 resistance and to streptomycin resistance, confirming the results of Luria and Delbrück.

Taken together, the evidence described above demonstrated the unequivocal existence of spontaneous mutations arising prior to and independently of exposure to selection. Experiments utilizing a chemostat went on to establish that spontaneous mutation rates were, for the most part, generation-dependent, suggesting that spontaneous mutations arose by errors in DNA replication (Novick and Szilard, 1950; Novick and Szilard, 1951). That mutations were due to spontaneous errors in DNA replication and arise without regard to utility became the mutational paradigm for about the next 40 years. It appeared as if Lamarckism had once and for all been discredited.

However, whereas the evidence was convincing, there was a problem with the experiments in that the selections used were lethal. Because phage T1 kills sensitive cells as does streptomycin, the selections were incapable of detecting specifically induced mutation. After all, dead cells cannot mutate (or at least such mutants would remain undetected). Consequently, although the above experiments proved that random mutation occurs, they failed to address properly the question of whether mutations can occur in response to exposure to

a selective environment. Max Delbrück seemed to recognize this, and made the following comments in a discussion of a paper by André Lwoff (1946):

"I would... like to make a point with respect to the use of the word 'spontaneous' as applying to mutations. The word merely means that certain factors which might be suspected of causing the mutations were shown to be without effect... In the case of mutations of bacteria from phage-sensitivity to phage-resistance... it could be shown that the phage does not cause the mutations. ... in your case of mutations permitting the mutants to utilize succinate... it seems to me an obvious question to ask whether this particular medium had an influence on mutation rate, and as long as this has not been ruled out the designation 'spontaneous' would seem improper. In view of our ignorance of the causes and mechanisms of mutations, one should keep in mind the possible occurrence of specifically induced adaptive mutations..."

Evidence for the existence of adaptive mutations

John Cairns and colleagues (1988) re-initiated the debate over Lamarckian and Darwinian evolution when they published an article in the journal *Nature* which provided evidence for the existence of "adaptive¹" mutations in bacteria. Cairns *et al.* (1988) recognized the limitations of the lethal selection systems used by others and described a system in which *Escherichia coli* cells when exposed to a

¹ The term adaptive as it refers to mutation in response to selection, is used predominantly in the literature and has historical precedence (Lwoff, 1946). As such, it will be used in this thesis as will stationary-phase. However, these mutations have been referred to by other names such as "directed" (Cairns, *et al.*, 1988), "anticipatory" (Hall, 1988; Symonds, 1989), "substrate-induced" (Davis, 1989), "Cairnsian" (Hall, 1990), "selection-induced" (Hall, 1992), "stressful-lifestyle associated" (Rosenberg, *et al.*, 1994), and stationary-phase (chapter 2; Torkelson *et al.*, 1997).

non-lethal genetic selection, (1) mutate in response to the selection, (2) do so in the apparent absence of cell-division, and (3) appeared to produce only mutations in the locus whose function is being selected. Although this is the most recently cited resurgence of Lamarckian mutation (but see Shapiro, 1984), Francis Ryan also provided evidence for the existence of adaptive mutation (Ryan, 1952; Ryan and Wainwright, 1954). Unfortunately, at the time these studies by Ryan went largely unheeded.

While experiments were being conducted that support the random spontaneous mutation hypothesis, Francis Ryan was discovering mutations in *E. coli* that appeared to be induced by the selection (reviewed by Ravin, 1976). Ryan (1952) examined the distribution of the numbers of lactose-utilizing (Lac^+) mutants among replicate cultures of a *lac*⁻ strain of *E. coli* K-12, after plating the *lac*⁻ cultures onto medium with lactose as the sole carbon source. Ryan observed that the distribution of mutants did not conform to a Luria and Delbrück distribution, but in fact produced a Poisson distribution. Ryan did not interpret his result as support for the existence of adaptive mutation, but attributed it to artifact.

Ryan and Wainwright (1954) discovered further evidence for the existence of adaptive mutations when they observed that stationary-phase cultures of *E. coli* auxotrophic for histidine (His^-), continued to produce His^+ prototrophs for a period of 10 days after the cells were inoculated into histidine-less media. The authors reasoned that these could be the result of stationary-phase mutation.

Ryan pursued this discovery and went on to show that when His^- *E. coli* cells are plated on agar medium lacking histidine, His^+ mutant colonies continue to appear over time (Ryan, 1955; Ryan, 1959; Ryan *et al.*, 1963). The mutants arising 2 to 3 days post-plating generated a distribution with a large variance

resembling the results of Luria and Delbrück, however, later arising mutants did not show a clonal origin, suggesting that they could have arisen after exposure to the selection (Ryan, 1955). In an attempt to explain these results, Ryan examined and discarded a number of possibilities (Ryan, 1955; Ryan, 1959; Ryan *et al.*, 1963; Ryan, *et al.*, 1961). Ryan was able to show that the mutations arising during starvation on histidine-less medium were not caused by phenotypic lag (Ryan, 1955), slowly growing mutants (Ryan, 1955), cell growth and division (Ryan, 1955; Ryan, 1959), or by cell turnover (Ryan, 1959; Ryan *et al.*, 1963). Finally, Ryan invoked the possibility of DNA replication without cell division (Ryan, *et al.*, 1961). Although Ryan could detect DNA synthesis in non-dividing stationary-phase bacteria, the amount of DNA synthesis that was taking place could not account for the amount of mutation he was observing (Nakada and Ryan, 1961; Ryan, *et al.*, 1961). Therefore, Ryan suggested that the mutations could be due to a small amount of error-prone DNA synthesis or to minor and selective base substitutions (Ryan, *et al.*, 1961).

Ryan's work was the first well-documented examination of what appeared to be adaptive mutation in bacteria. Nevertheless, Ryan's work went largely unnoticed until the 80's, when the ghost of Lamarck again reared its head.

In 1984, further evidence for the existence of adaptive mutations appeared. James Shapiro was studying a previously described system in which a strain of *E. coli*, carries the regulatory region of the arabinose operon separated from the carboxy-terminus of the *lacZ* gene by a Mu prophage. In this system the *ara* operon can be fused to *lacZ* upon excision or deletion of prophage Mu (Casadaban, 1976; Shapiro, 1984). The strain Shapiro used is both Lac⁻ and Ara⁻ [identified as Lac(Ara)⁻]. If the Mu bacteriophage is excised such that *lacZ* and *araB* are fused in frame, the strain can utilize lactose when arabinose is present to

induce transcription of the fusion gene. Shapiro observed that when Lac(Ara)⁻ cells are plated on lactose-arabinose minimal medium at 32°C, Lac(Ara)⁺ mutants arise on the plate after 5 to 6 days and continued on average for 20 days, at which point the rate of mutant formation declined (Shapiro, 1984). Reconstruction experiments, in which independent fusion clones were subcloned onto minimal lactose-arabinose agar revealed that growth into colonies occurs in 2 days at 32°C. Similarly, if a Lac(Ara)⁺ fusion was transduced into cells, colonies appeared on this medium within 2 days, suggesting that the rate limiting step is the Mu deletion event. The Lac(Ara)⁺ fusion mutants do not arise during starvation in the absence of selection (i.e.: the absence of lactose) and are undetectable during non-selective growth prior to plating. Thus, the Lac(Ara)⁺ fusion mutants appear only under selection, suggesting that this may be another example of adaptive mutation. More recently, the *ara lac* fusions have been obtained non-selectively (Mittler and Lenski, 1990; Maeneut-Michel and Shapiro, 1994; Foster and Cairns, 1995). However, the fact that fusion structures differ in the selectively and non-selectively obtained fusions (Maeneut-Michel and Shapiro, 1994) indicates that a different fusion mechanism operates during selection, in support of the existence of a special "adaptive" or at least physiologically controlled mechanism.

The ground breaking study that has led to further investigation was performed by John Cairns *et al.* (1988), who had recognized the limitations of the classical experiments described above. First, they examined reversions to Lac⁺ after plating multiple cultures of *E. coli lacZ*_{am} on minimal lactose plates (Cairns, *et al.*, 1988). The strain carries the *lacZ*_{am} allele on its F' episome, and the chromosomal *pro-lac* region and *uvrB-bio* region are deleted. Cairns *et al.* (1988) observed that Lac⁺ revertants accumulated at a constant rate over time after

exposure to selection. The distribution of the revertants was intermediate between a Luria and Delbrück distribution and the Poisson. The authors suggested that this could represent both random spontaneous mutation that occurred prior to plating, during the growth of the culture, and post-plating mutation, in response to exposure to selection. In addition, they observed that Lac⁺ revertants do not accumulate in the absence of selection. To address the possibility of slow growth of the Lac⁻ cells on the plate (perhaps due to the *lacZ*am allele being leaky or to impurities in the medium), Cairns and colleagues tested for the presence of another rapidly expressed mutation, mutation to valine resistance (Val^R). They observed no concomitant accumulation of unselected Val^R mutants. The authors suggested,

"that populations of bacteria, in stationary-phase, have some way of producing (or selectively retaining) only the most appropriate mutations."²

Second, John Cairns and colleagues tested the system used by Shapiro in which the deletion of a Mu prophage fuses *araB* and *lacZ* (Cairns, *et al.*, 1988; Shapiro, 1984). Cairns and colleagues also observed the accumulation of Lac(Ara)⁺ mutants over time, confirming the results of Shapiro. In addition, they observed that in rich media, without lactose or arabinose, Lac(Ara)⁺ mutants do not accumulate at a detectable rate, but when lactose is added to a stationary-phase culture that has arabinose but no lactose, Lac(Ara)⁺ mutants begin to accumulate. The authors concluded, that --

² It has since been shown that the vast majority of late-arising Lac⁺ mutants are slow growers and probably existed prior to plating (Prival and Cebula, 1996). However, although this particular example of adaptive mutation now appears to be invalid, the Cairns *et al.* (1988) paper stimulated a number of scientists to further examine the phenomenon of adaptive mutation with more success.

" This therefore seems to be another example of the production of appropriate mutations in response to selection."

Finally, Cairns and colleagues noted the fact that *E. coli* deleted for *lacZ* can acquire the ability to ferment lactose (Hall, 1982). *E. coli* harbors a cryptic gene *ebgA*^o, that can be activated by two point mutations. One is in the repressor gene *ebgR* and the other is in the *ebgA* gene itself, which encodes a unique lactose hydrolyzing enzyme (Hall, 1982). The rate of each of these point mutations, during growth, is less than 10^{-8} (Hall, 1982). Therefore, if the mutations occur independently of each other, the frequency of generating both to produce a Lac⁺ cell should be 10^{-16} . Yet colonies that have grown to stationary-phase on a plate containing lactose, will generate Lac⁺ papillae after about 2 weeks. Hall has shown that the frequency of mutation to Lac⁺ using the *ebgA* route is 10^{-8} (Hall, 1982). Cairns and colleagues suggest that this represents another example of adaptive mutation (Cairns, et al., 1988).

7 Cairns *et al.* (1988) by no means thoroughly examined these proposed cases of adaptive mutation. The result of the paper however, was much stimulated discussion and debate (e.g.: Benson, 1988; Cairns, *et al.*, 1988; Charlesworth, *et al.*, 1988; Danchin, 1988; Holliday and Rosenberger, 1988; Lenski, *et al.*, 1989; Partridge and Morgan, 1988; Stahl, 1988; Tessman, 1988; Van Valen, 1988). The study by Cairns and colleagues sparked further experimentation on the existence and possible mechanism(s) of adaptive mutation, and has ultimately led to a much greater understanding of the complexity of mutational variation (below and this work).

Barry Hall followed Cairns *et al.* (1988) in describing two additional examples of adaptive mutation (Hall, 1988; Hall, 1990). First, Hall studied a system which utilized a strain of *E. coli* that is unable to metabolize salicin unless

two specific mutational events occur (Hall, 1988). The *bgl* (β -glucoside) operon of this *E. coli* K-12 strain is cryptic and requires two mutations for activation, namely a point mutation in *bglR*^o to generate the functional *bglR*⁺ which allows for transcription of the *bgl* structural genes and, an IS element excision in the *bglF*::IS103 allele to restore normal function to *bglF* and allow for transport of salicin into the cell. The spontaneous rates of these two mutations are $2-6 \times 10^{-8}$ and $< 2 \times 10^{-10}$, respectively (Parker, *et al.*, 1988). Therefore, assuming the mutations are independent events, the expected rate of forming the double mutant should be on the order of 10^{-18} . Hall observed, however, that in 2-3 week old colonies on MacConkey salicin plates, the double mutants (Sal⁺) occurred at frequencies of 10^{-8} , while the unselected mutation rate to valine resistance was unaffected (Hall, 1988). In addition, Sal⁺ mutants were not detected when cells were incubated for 4 weeks on MacConkey medium lacking salicin.

Following this first example of adaptive mutation, Hall studied reversions of *E. coli* tryptophan auxotrophs (Trp⁻) to the prototrophic condition (Trp⁺) that occur more frequently when the event is advantageous than when the event is neutral (Hall, 1990). Hall found that revertants (identifiable by the presence of Trp⁺ papillae) of two strains bearing point mutations in *trpA* or *trpB* continued to appear for 8-10 days after plating on medium lacking tryptophan, and the distribution of these late arising mutants conformed to the Poisson distribution. Reconstruction experiments indicated that a Trp⁺ cell forms a visible papilla in 4 days. Hall was able to eliminate the artifactual explanations of cryptic growth of cells within colonies, and phenotypic lag. Furthermore, Hall demonstrated that in the absence of selection both Trp⁺ and Cys⁺ papillae fail to accumulate, as well the overall mutation rate (as measured by mutation to Val^R and mutation to

lacI⁻) was not increased during tryptophan starvation. Thus, this appeared to be an example of adaptive mutation.

Although *E. coli* is the most often used organism for studying adaptive mutation, examples of adaptive mutation in yeast have been described as well (Hall, 1992; Steele and Jinks-Robertson, 1992). Hall (1992) found that reversions of a histidine auxotroph (again scored as papillae), arose six days after plating on histidine-less media and continued for up to 23 days. These cells did not simultaneously accumulate a requirement for inositol, suggesting to Hall that reversion to His⁺ was specific to the selection. Hall (1992) showed that the cells were not actively growing. Steele and Jinks-Robertson (1992) studied reversion to Lys⁺ prototrophy of a strain containing a *lys-2* frameshift mutation. The authors demonstrated that Lys⁺ revertants accumulate in a time-dependent manner (as opposed to growth-dependent) and the distribution of late arising colonies was consistent with the Poisson (Steele and Jinks-Robertson, 1992). They observed that when these cells were starved for other amino acids singly or together with lysine, that there was no accumulation of Lys⁺ revertants (Steele and Jinks-Robertson, 1992). The authors suggest then, that this represents an example of adaptive mutation in yeast (Steele and Jinks-Robertson, 1992).

The studies discussed above appear to bear 3 commonalities: (i) the mutations occur in apparently non-dividing cells; (ii) they seem to arise only after exposure to selection, and (iii) have been detected so far only in genes whose functions were selected (but see Hall, 1990). What is missing from these studies is information on the molecular mechanism behind the mutation being observed. One experimental system has proven useful in working out a molecular mechanism behind some adaptive mutations. This system is the focus of this thesis and is used in all experiments presented.

The *lacI33 Ω lacZ* frameshift system

The *lac* frameshift system described by Cairns and Foster (1991) uses a strain of *E. coli* deleted for the chromosomal *lac* operon and harboring a *lacI33 Ω lacZ* fusion allele on the F-episome. There is a frameshift (CCC to CCCC) in *lacI* which is polar on *lacZ* conferring a Lac⁻ phenotype. The fusion allele is constitutively expressed from the *lacI_P* promoter. This strain is particularly useful for genetic analysis as it has a high rate of reversion to Lac⁺ when under conditions of selection. When cells are plated on minimal lactose plates, in the presence of an excess of male, non-revertible scavenger cells, which consume any contaminating carbon sources, Lac⁺ colonies appear continuously over time. Those mutants arising early after plating (2 days) are growth-dependent revertants and form a Luria and Delbrück distribution. The distribution of later arising mutants tends more towards the Poisson, suggesting that the later arising mutants arise only after exposure to selection (Cairns and Foster, 1991). Approximately 95% of the colonies appearing in the first week after plating are the result of mutations that occur after the cells had been spread on lactose-minimal plates (Cairns and Foster, 1991). As Lac⁺ revertants appear, the total number of viable *lac*⁻ cells remains unchanged (Cairns and Foster, 1991). The Lac⁺ adaptive mutants do not arise in the absence of lactose, or in the presence of lactose if there is another unfulfilled requirement for growth, and appear not to be due to cryptic growth of the *lac*⁻ frameshift-bearing cells (Cairns and Foster, 1991; Foster, 1994). Finally, the selective conditions are not acting as a non-specific mutagen because an unselected mutation failed to accumulate during lactose selection (Foster, 1994).

Molecular handles on the *lacI33ΩlacZ* frameshift system

Hastings and Rosenberg (1992) proposed a possible mechanism by which non-growing cells, which may not be replicating their DNA, could generate mutations. They suggested that a mechanism analogous with somatic hypermutation of immunoglobulin variable region genes in chickens (Hastings and Rosenberg, 1992). The immature B cells of chicken achieve V-region diversity by recombination between partially homologous sequences (Maizels, 1987). Recombination has since been shown to be involved in adaptive mutation (Harris, *et al.*, 1994), though not by this mechanism.

The major system of homologous recombination in *E. coli* is the RecBCD system (reviewed by Rosenberg and Hastings, 1991). This system requires RecA protein, the RecBCD holoenzyme, Holliday junction resolvases, DNA double-strand breaks (DSBs), and a unique eight base pair sequence called Chi. In homologous recombination the RecBCD enzyme creates single-strand DNA allowing RecA protein to catalyze strand invasion of a homologous duplex DNA molecule, forming a Holliday junction (see Rosenberg and Hastings, 1991). The RecA and RecBC proteins also function in the SOS pathway of DNA damage repair (reviewed by Walker, 1996). RecA protein is activated by a signal, probably single-stranded DNA, and becomes a co-protease which helps cleave the LexA protein. The LexA protein is a repressor of many genes (including *recA*) whose products function in DNA damage repair (reviewed by Walker, 1996). That one LexA regulated gene, other than *recA*, is required for full levels of adaptive mutation is apparent because a strain bearing an operator constitutive allele of *recA* (*recA_{o281}*) and an uncleavable Lex repressor allele (*lexA3*) displays decreased levels of adaptive mutation (R.S. Harris and S.M.

Rosenberg, personal communication). However, the discovery that additional recombination proteins are required for adaptive mutation indicates that a major role of the RecA and RecBC proteins is in recombination (Foster, *et al.*, 1996; Harris, *et al.*, 1996).

RecBCD is a heteromultimeric enzyme with subunits encoded by the *recB*, *recC*, and *recD* genes (Taylor, 1988). The enzyme is also called exonuclease V and has both helicase and single-strand endonuclease activity, which together constitute its double-strand exonuclease activity (Taylor, 1988). Rosenberg and Hastings (1991) proposed the split-end model for RecBCD-mediated homologous recombination. In this model, RecBCD loads exclusively onto DNA DSBs and travels along the DNA acting as exonuclease V until it reaches a sequence of nucleotides called Chi. At this eight base pair sequence, the RecD subunit is lost and the RecBC enzyme then acts as a helicase, unwinding the DNA creating single strands. This single-stranded DNA substrate can then be coated with RecA protein, which catalyzes strand invasion of a homologous duplex DNA molecule. The RecD protein is required for the RecBCD holoenzyme's nucleolytic activity, and *recD* null mutants have a hyper-recombinagenic phenotype (Amundsen, *et al.*, 1986; Biek and Cohen, 1986; Thaler, *et al.*, 1989). Null mutations in *recB* or *recC* destroy all function of the RecBCD enzyme and render cells recombination-deficient (see Taylor, 1988).

The requirements for adaptive mutation in *E. coli* parallel those for homologous recombination in the RecBCD pathway (Harris, *et al.*, 1994). Recombination-deficient *recA* and *recB* cells show no adaptive mutation, and hyper-recombinagenic *recD* cells are hypermutable (Harris, *et al.*, 1994). The hypermutable phenotype of *recD* cells is *recA*- and *recB*-dependent (Harris, *et al.*, 1994) similar to recombination. Growth-dependent rates of mutation are

unaffected by mutations in *recA*, *recB*, *recC*, or *recD* (Cairns and Foster, 1991; Harris, *et al.*, 1994). This implies the involvement of homologous recombination in formation of adaptive mutations. Furthermore, genes whose products process Holliday junction intermediates are also required for adaptive mutation (Foster, *et al.*, 1996; Harris, *et al.*, 1996). Harris *et al.* (1996) and Foster *et al.* (1996) find that null mutations in *ruvA*, *ruvB*, or *ruvC* abolish adaptive mutation. The requirement for RecBCD implies that DSBs are an intermediate in the formation of adaptive mutations, as RecBCD loads exclusively at DSBs (see Taylor, 1988). As well, the requirement for Holliday junction resolution enzymes implies that recombination intermediates are also intermediates in the formation of adaptive mutations.

Harris and colleagues (1994) proposed 2 classes of models to explain the role of recombination in adaptive mutation. In templated models, mutant sequence(s) that restore Lac⁺ information preexist in the genome (Grafen, 1988; Hastings and Rosenberg, 1992). These sequences could be transferred into the mutating gene by recombination. In non-templated mutation models the mutant sequence is formed *de novo*. The templated mutation models predict a small number of possible reversion sequences and the occurrence of associated silent mutations, while non-templated models predict many possible reversion mutations and no association of silent, extraneous base changes (Rosenberg, *et al.*, 1994).

Rosenberg *et al.* (1994) and Foster and Trimarchi (1994) independently sequenced samples of Lac⁺ adaptive revertant isolates and found (i) that they are nearly all single base deletions in small mononucleotide repeats and, (ii) they are not associated with extraneous base changes. This contrasts with the growth-dependent Lac⁺ reversions which are heterogeneous, including small and large

deletions, insertions, and duplications. Both groups concluded that this sequence spectrum of Lac⁺ adaptive revertants supported non-templated models for adaptive mutation, and noted that the sequence spectrum is characteristic of polymerase template-slippage errors (Ripley, 1990). In fact, it has been shown that DNA polymerase III, the major replicative polymerase of *E. coli*, is involved in the formation of adaptive mutations (Foster, *et al.*, 1995; Harris, *et al.*, 1997a).

Both groups also noted that template-slippage mutations in simple repeats are characteristic of cells lacking post-synthesis mismatch repair (Levinson and Gutman, 1987; Parsons, 1993; Strand, *et al.*, 1993), and postulated that the hypermutability of *E. coli* cells exposed to selection could indicate decreased mismatch repair. Down-regulation of mismatch repair during adaptive mutation had been suggested but not in the context of recombination-dependent, non-templated, adaptive mutations (Stahl, 1988; Hastings and Rosenberg, 1992; Jayaraman, 1992).

The sequence spectrum of growth-dependent Lac⁺ revertants isolated from mismatch repair null mutant strains is identical to the adaptive reversion sequence spectrum, supporting the hypothesis of suspended mismatch repair during adaptive mutation (Rosenberg *et al.*, 1994). However, most Lac⁺ revertants do not possess a heritable mutator phenotype (Longerich *et al.*, 1995; and this work). Mismatch repair appears to be down-regulated during adaptive mutation by a transient deficiency in functional MutL protein (Harris, *et al.*, 1997b).

Finally, F-episome transfer (Tra) functions are required for Rec-dependent adaptive reversion of the F' located *lac* gene, however actual transfer of the F is not required (Foster and Trimarchi, 1995a; Foster and Trimarchi, 1995b; Galitski and Roth, 1995). It has been suggested that transfer synthesis generates the

adaptive Lac⁺ reversions (Galitski and Roth, 1995; Peters and Benson, 1995; Radicella, *et al.*, 1995), however it is possible that the role of the Tra proteins in adaptive mutation is nicking at *oriT* (the origin of transfer of the F) which could lead to DSBs allowing RecBCD to load and initiate formation of recombination intermediates from which DNA synthesis could be primed (Rosenberg, *et al.*, 1995).

Is it directed?

The most controversial question underlying the phenomenon of adaptive mutation pertains to Lamarck versus Darwin. Is adaptive mutation directed exclusively to the gene under selection? If this were the case, then cells under selection should not accumulate neutral mutations. This is a testable hypothesis and researchers have attempted to address this question in several systems (Cairns and Foster, 1991; Cairns, *et al.*, 1988; Foster, 1994; Hall, 1988; Hall, 1990; Hall, 1992; Steele and Jinks-Robertson, 1992).

Mutation to valine resistance (Val^R) has been used as a neutral unselected phenotype in several studies (Cairns, *et al.*, 1988; Hall, 1988; Hall, 1990). *E. coli* K-12 strains are sensitive to exogenous valine which acts as a false feedback inhibitor of isoleucine biosynthesis. Mutations at several loci can generate Val^R; thus it acts as a large mutational target (De Felice, *et al.*, 1979). However, the validity of using Val^R has been questioned (Danchin, 1988; reviewed by Drake, 1991; reviewed by Foster, 1993; Partridge and Morgan, 1988). In fact, the mutation rate to valine resistance varies and is considerably lower than expected for even a single gene in different K-12 strains (Hall, 1988; Hall, 1990).

Hall (1990) showed that when *trpB*⁻ or *cysB*⁻ cells (which continuously revert to the prototrophic condition if exposed to selective media) were placed on medium limited only for the other amino acid and incubated for 40 hours, they did not accumulate revertants. Hall concluded that no Trp⁺ mutants formed on cysteine limited medium and no Cys⁺ mutants formed on tryptophan-limited medium. However, 40 hours may not have been enough time to allow cells, starved for one amino acid but given another, to switch their biosynthetic pathways in time to produce visible papillae (Foster, 1992).

In the *lacI33ΩlacZ* frameshift system Foster (1994) found that when a rifampicin sensitive derivative of the *lac*⁻ frameshift bearing strain is plated on minimal lactose plates it does not display an increase in rifampicin resistance (Rif^R). Foster (1994) concluded that there does not appear to be a general increase in the mutation rates under selective conditions. However, Foster conceded that the mutagenic process that occurs could affect only particular mutations or certain regions of the chromosome. The mutational events that give rise to Rif^R are simple base-substitutions in the gene encoding the β subunit of RNA polymerase (Ovchinnikov, *et al.*, 1983). Consequently, this system may be frameshift-specific or region specific and mutations that require substitutions may be inappropriate as unselected mutational targets (chapter 2; Torkelson *et al.*, 1997).

For the *lacI33ΩlacZ* frameshift system, 2 sites in the chromosome are inactive for recombination-dependent Lac⁺ reversion (Foster and Trimarchi, 1995a; Galitski and Roth, 1995; Radicella, *et al.*, 1995). However, the fact that the molecular mechanism implies that DSBs are an intermediate in the formation of adaptive mutations, and that there is evidence that different chromosomal regions are differently accessible to DSBs (Louarn *et al.*, 1994), raises the

possibility that certain regions of the chromosome will be "cold" for adaptive mutation (reviewed by Rosenberg *et al.*, 1995, 1996). It is possible that the chromosomal loci tested up until now have been in regions that do not experience a high frequency of DSBs. One could predict that if these earlier studies had looked for mutation at multiple sites around the chromosome, a high frequency of mutation may have been observed at some but not all sites.

Although his data were few, Hall (1990) observed two unselected auxotrophic mutants amongst 110 Trp⁺ adaptive revertants. This gave a frequency of unselected mutation at least 18-fold greater than among other tryptophan-starved cells (Hall, 1990). Hall (1990) postulated that a fraction of starved cells in a colony enters a hypermutable state in which extensive DNA damage and resulting error-prone repair synthesis occurs, while the remaining cells in the colony remain essentially immutable. If one of the mutations that is occurring in a cell undergoing this hypermutation rescues the cell from starvation, then the cell could exit the hypermutable state and grow. If however, none of the mutations rescue the cell, then the cell will die. Hall suggested that when aged colonies are tested, mutations in loci not under selection could not be recovered because the only viable cells would be those that had never entered the hypermutable state. This predicts then, that the frequency of unselected mutations may increase but only amongst the selected mutants (Hall, 1990). It has since been suggested that the reason cells that enter a hypermutable state die could be through the continued formation of DSBs (Harris *et al.*, 1994; Rosenberg, 1994; Rosenberg, *et al.*, 1995; 1996).

Hall's (1990) hypermutable state hypothesis may serve to explain why previous studies failed to detect unselected mutation. These studies examined

the entire population of starving cells as opposed to looking specifically in the population that had experienced an adaptive reversion event.

Thesis objectives

The aim of this thesis is to address three questions. First and foremost, is adaptive mutation in this system directed in a Lamarckian manner exclusively to the *lac* gene under selection? Second, is the mutational process specific to the F episome? Third, an attempt is made to test the postulate of Hall (1990), which states that adaptive mutants are generated by a subpopulation of cells exposed to the selection, which experience a (random Darwinian) hypermutable state.

The data in Chapter 3 demonstrate unequivocally that adaptive mutation, in this system, is not directed in a Lamarckian manner to the *lac* gene under selection. Unselected genes, in all replicons in the cell, experience a hypermutable state. This along with data in Chapter 2 also demonstrates that adaptive mutation is not confined to the F-sex plasmid. Hypermutation was not detected in non-revertants exposed to selection, supporting Hall's (1990) postulate that a differentiated subpopulation of cells experiences a hypermutable state.

The demonstration of the existence of hypermutable subpopulations in starving and non-growing cells has implications not only in bacterial evolution, but in the generation of mutations in non-growing cell populations of multicellular organisms. This could be relevant to the generation of somatic mutation that can lead to cancer in animals.

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Chapter 2*

Is transfer necessary for adaptive mutation?

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Introduction

Debate has raged over the generality of the adaptive mutation mechanism in the system utilizing an F-born *lacI33 Ω Z* fusion allele. Is the mechanism specific to the molecule that the reverting gene resides on, or can it be generalized to post-selection mutagenesis in other replicons (Benson, 1997; Galitski and Roth, 1996; Radicella, *et al.*, 1995). This will be determined when the other post-selection mutagenesis systems are tested for the same molecular handles that are required for adaptive mutation in our system. However, in recent years, attempts at answering this question have been made using the F' *lac* frameshift system.

First, although the F' is a hot site for recombination-dependent Lac⁺ adaptive reversion, the native *lac* locus on the bacterial chromosome appears to be cold (Foster and Trimarchi, 1995; Radicella, *et al.*, 1995), suggesting that location relative to some other DNA site influences adaptive reversion (either positively or negatively). That site could be the transfer origin of the F'.

Two groups have suggested a necessary link between conjugative transfer and adaptive reversion of the F' *lac* (Peters and Benson, 1995; Radicella, *et al.*, 1995). In adaptive mutation experiments, the *lac* frameshift-bearing cells are plated in the presence of an excess of *lac* deletion (non-revertible) "scavenger" cells that consume any residual non-lactose carbon sources that might be present which the frameshift-bearing cells could utilize to grow and divide (Cairns and Foster, 1991). The frameshift-bearing cells are male with the reverting *lac* allele on their F'. The scavengers are also male (a presumed deterrent to conjugation) but carry no *lac* genes. Peters and Benson (1995) and Radicella *et al.* (1995) found that these male cells have some ability

to mate when they are starving on lactose medium. The F' of the frameshift cell occasionally ends up in the scavenger. Whether this happens more often when Lac⁺ reversion occurs than when it does not was reported by neither group. Thus sexual transfer was not correlated (positively or negatively) with the mutations.

Adaptive mutation in the F' *lac* system requires the homologous recombination gene *recA* (Cairns and Foster, 1991; Harris *et al.*, 1994). In order to determine if transfer of the F is necessary for adaptive mutation, I tested whether the *rec* genotype of the male scavenger cells has any affect on mutation rate.

Results and discussion

Some evidence discourages the idea of transfer being necessary for adaptive mutation:

First, only 10% or fewer of the Lac⁺ revertants carry a marker from the chromosome of the scavenger (Radicella *et al.*, 1995; and data to follow). I assayed 116 Lac⁺ adaptive revertants isolated previously (Rosenberg, *et al.*, 1994) for rifampicin-resistance, a chromosomal marker present in the frameshift-bearing cells, but absent from the chromosome of the scavenger cells. Nine out of 116 are rifampicin-sensitive, indicating that the revertible F' transferred to a neighboring scavenger cell in only about 8% of reversions.

Second, it is likely that these Lac⁺ mutations arose in the frameshift-bearing cell and were transferred subsequently to the scavenger because *rec* mutations in the frameshift-bearing cell, that are not in the scavengers, abolish adaptive mutation (Harris *et al.*, 1994). Here I report that the *rec*

genotype of the scavenger does not affect adaptive mutation rates (Fig. 2-1), implying that reversion occurs in the frameshift-bearer, not in the scavenger cell. Thus, actual transfer seems unlikely to be necessary for adaptive mutation (also see Foster and Trimarchi, 1995, discussed below).

Although F' transfer seems unnecessary for reversion of the frameshift allele, transfer functions facilitate recombination-dependent *lac* reversion when the frameshift allele is carried on the F'. Galitski and Roth (1995) moved the F' in question into *Salmonella*. In *Salmonella*, repression of the transfer genes inhibits adaptive Lac reversion. In *E. coli*, mutations in *traD* or *traQ* genes diminish adaptive Lac reversion by a factor of ten (Foster and Trimarchi, 1995). But because conjugation is reduced by five orders of magnitude by *traD* and *traQ* mutations, these results show a failure to correlate conjugative transfer-proficiency and *lac* adaptive mutation. However, the transfer proteins are important, suggesting that their action at *oriT*, the sexual replication and transfer origin, is required.

What role do the Tra proteins play in adaptive Lac reversion? The Tra proteins do more than one thing at *oriT*. They make a single-strand nick at that site, and then that nick is used by them to initiate DNA synthesis (Frost, *et al.*, 1994). Galitski and Roth (1995) propose that the synthesis is the relevant part for adaptive mutation (see also Foster and Trimarchi, 1995). They suggest that polymerase mistakes made during that synthesis are the origin of the mutations, and that the uni-directionality of sexual synthesis produces the unique mutation spectrum of all -1 deletions in mononucleotide repeats. Because that odd sequence spectrum can be reproduced by removal of mismatch repair during bi-directional replication (growth) (Longerich, *et al.*, 1995), their hypothesis seems unlikely. In addition, the idea that transfer synthesis of the F' is necessary for adaptive mutation and the idea that

mutation in this system is specific to the F' predicts that any unselected mutation associated with Lac⁺ reversion on the F' would itself be confined to the F'. The results described in chapter 3 of this work demonstrate unselected mutation in association with Lac⁺ reversion on all replicons in the cell. This discourages transfer synthesis models. It is possible, instead, that the nicks at *oriT* develop into DSBs, the same DSBs that must be present to allow RecBCD participation (Harris *et al.*, 1994; Rosenberg, *et al.*, 1996; Longerich *et al.* 1995). Those DSBs could be used by the recombination proteins to initiate recombination intermediates from which DNA synthesis is primed.

The implication of this idea is that any DSB site that gets activated during starvation will promote adaptive mutations nearby. The native *lac* locus could be cold because it is distant from such sites. But this does not discourage the possibility of chromosomal hotspots for recombination-dependent adaptive mutation that occurs using a mechanism very similar to that used on the F'. Good candidates for such sites are the terminus of replication, a RecBCD-dependent recombination hotspot (Louarn, *et al.*, 1991), the *oriM* sites at which RecBC-dependent "stable" DNA replication is initiated (Asai, *et al.*, 1993), and there may well be others that are exclusive to starvation conditions.

This question will be resolved when an F-independent site is demonstrated to undergo Rec-dependent mutation similar to that described on the F', or when the search for such sites has been exhausted.

Materials and Methods

Experiments were performed as described previously (Harris *et al.*, 1994) using $\Delta(srlR-recA)306::Tn10$, the null allele employed in that work. Scavenger cells were plated in 25-fold excess relative to the frameshift-bearing cells in the same top agar layer. Daily viable cell counts of total *lac*⁻ frameshift-bearing cells were performed as described previously (Harris *et al.*, 1994).

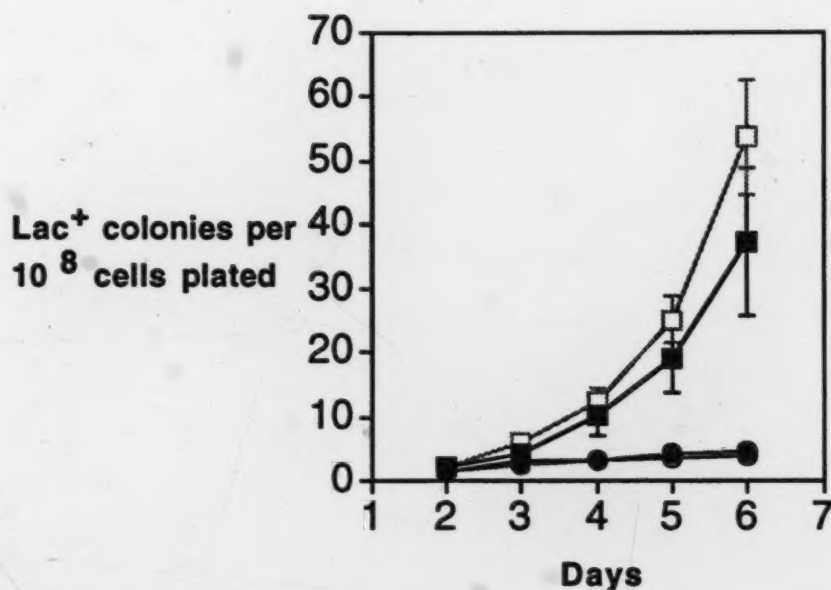


Figure 2-1. A *recA* null mutation abolishes adaptive mutation when it is in the frameshift-bearing cell, and not when it is in the scavenger cell. Discussed in the text. The following combinations are shown: *rec*⁺ frameshift-bearing cells plated in the presence of *rec*⁺ scavenger cells (closed squares); *rec*⁺ frameshift-bearing cells plated with *recA* scavengers (open squares); *recA* frameshift-bearing cells plated with *rec*⁺ scavengers (closed circles); *recA* frameshift-bearing cells plated with *recA* scavengers (open circles). Error bars represent one standard error of the means. As previously, daily viable cell counts of total *lac*⁻ frameshift-bearing cells showed no net growth or death of the population.

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Chapter 3*

Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation

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Introduction

"Adaptive" mutations are an unexpected kind of spontaneous mutation that is distinguished from normal spontaneous growth-dependent mutations (e.g. Luria and Delbrück, 1943; Lederberg and Lederberg, 1952) by the criteria that adaptive mutations are detected only in the presence of selection for those mutations, and in non-dividing or slowly-growing stationary-phase cells (e.g. Ryan, 1955; Cairns *et al.*, 1988; Cairns and Foster, 1991; Jayaraman, 1992; Hall, 1990; Foster, 1994; Steele and Jinks-Robertson, 1992; Hall, 1992; reviewed by Foster, 1993; Drake, 1991). Adaptive mutation provoked controversy about whether mutagenesis mechanisms exist that direct mutation preferentially to a selected gene, in a Lamarckian manner (e.g. Cairns *et al.*, 1988; Charlesworth *et al.*, 1988; Lenski and Mittler, 1993a; 1993b; Cairns, 1993). The unorthodoxy of this suggestion led many to argue that adaptive mutation must not exist (reviewed by Foster, 1993; Culotta, 1994). In one experimental system, adaptive mutation has been demonstrated to exist as a process mechanistically distinct from growth-dependent mutation by the following findings: Adaptive reversion of a *lac* frameshift mutation carried on an F' sex plasmid in *Escherichia coli* (Cairns and Foster, 1991) requires homologous genetic recombination functions (Harris *et al.*, 1994, 1996; Foster *et al.*, 1996), and produces a novel sequence spectrum (Rosenberg *et al.*, 1994; Foster and Trimarchi, 1994). Neither of these is seen in growth-dependent reversions of the same allele. These findings demonstrate a novel mutagenic mechanism for the adaptive *lac* reversions which includes genetic recombination and has other unique features (reviewed by Rosenberg, 1994; Rosenberg *et al.*, 1995, 1996; and discussed below). However, it has been suggested that this mechanism may represent a peculiarity of sex plasmid biology which would not pertain to mutagenesis in general, and would not

operate on replicons other than sex plasmids (Radicella *et al.*, 1995; Galitski and Roth, 1995; Foster and Trimarchi, 1995; Peters and Benson, 1995; and discussed below).

In this chapter I address three questions: First, is the mutagenic process that produces recombination-dependent Lac⁺ adaptive mutations directed in a Lamarckian manner preferentially to the *lac* gene? Second, is that mutation mechanism specific to the F' sex plasmid? And third, I test the postulate of Hall (1990) that adaptive mutants arise from a differentiated subpopulation of all the cells exposed to selection which experiences a (random Darwinian) genome-wide hypermutable state (also considered by Ninio, 1991; Harris *et al.*, 1994; Rosenberg, 1994). First, it is found that the mutational process that generates the Lac⁺ adaptive reversions is not directed to the *lac* genes, and second, that it is not specific to the F' replicon that carries *lac*. Unselected genes in all replicons in the cell are mutated at a high level in association with Lac adaptive reversion. Third, genome-wide hypermutation underlying adaptive Lac reversion is found to occur in a subpopulation of cells exposed to selection as suggested (Hall, 1990). These findings bear on the molecular mechanism of recombination-dependent adaptive mutation and on its evolutionary significance.

Results

Assay for a hypermutable subpopulation

To test the possibility that random, genome-wide mutation might occur in only a small subpopulation of the cells exposed to selection (Hall, 1990; Ninio, 1991; Harris *et al.*, 1994), a replica-plating screen was used to score unselected mutations. Unselected mutations were scored amongst Lac⁺ adaptive revertant

colonies and in two control populations (Materials and methods): the Lac⁻ frameshift-bearing cells grown into colonies without exposure to selection (Lac⁻ unstressed colonies), and colonies derived from the Lac⁻ frameshift-bearing cells that were exposed to selection, but which did not mutate to Lac⁺ (Lac⁻ stressed cell colonies). To obtain Lac⁺ adaptive revertants and Lac⁻ stressed cells, the *lac* frameshift-bearing cells were plated on lactose minimal medium and incubated for six to seven days as described (Cairns and Foster, 1991; Harris *et al.*, 1994, 1996). Growth-dependent Lac⁺ mutant colonies appear after two days incubation and are followed by the appearance of RecBC-dependent adaptive revertants on days 3-7 (Cairns and Foster, 1991; Harris *et al.*, 1994, 1996; Foster *et al.*, 1996; Rosenberg *et al.*, 1994; Foster and Trimarchi, 1994). The Lac⁻ stressed cells were rescued from the plates after 4-6 days of incubation by resuspending plugs of agar and plating to form colonies on medium containing a utilizable carbon source. The Lac⁺ adaptive revertant colonies, and colonies of the Lac⁻ stressed cells, and of Lac⁻ unstressed cells were replica-plated to assay mutations in several other genes in three replicons in the cells: a pBR322-derived plasmid, the F', and the bacterial chromosome. The Lac⁻ stressed and unstressed cell colonies were grown to be at least as large as the largest Lac⁺ adaptive revertant colonies replicated so that any higher frequencies of unselected mutation detected amongst Lac⁺ adaptive revertants could not have arisen trivially because of more cell generations during colony growth. This point is also demonstrated in experiments below.

Unselected mutations in a plasmid

Reversions of mutant tetracycline-resistance genes (Tet genes) on pBR322-based plasmids were examined. Four different plasmids were used as mutation targets. Each is identical with pBR322 except for a frameshift mutation that inactivates

the Tet gene (Table 3-1). Lac⁺ adaptive revertants, and Lac⁻ unstressed and stressed cells prepared from strains carrying each of the plasmids were grown into colonies and replica-plated to rich medium containing tetracycline. The data in Table 3-1 show that two of the Tet alleles tested revert frequently enough to score in this assay, and their reversion is 10-100 or more times higher amongst Lac⁺ adaptive revertants than in the two Lac⁻ control populations. The hypermutation is seen amongst adaptive Lac⁺ revertants but not amongst Lac⁻ cells from the same starved cultures. This implies that a subpopulation of cells exposed to starvation on lactose experiences mutability that can affect a gene in another replicon. These data demonstrate a strong correlation between Lac⁺ adaptive reversion and reversion of an unselected gene.

A sample of adaptive mutation-associated Tet^R mutations was mapped to the pBR322-based plasmid, and shown not to be associated with the F' that carries the *lac* genes. Plasmid DNAs prepared from 10 independent Tet^R isolates from the strain carrying plasmid pW17 (Table 3-1) were transformed into a plasmid-free female strain of *E. coli*, the parent of the *lac* frameshift-bearing strain (Cairns and Foster, 1991) and tetracycline-resistance was selected. In all cases, tetracycline-resistance was transferred to the new cells (as was ampicillin-resistance which is also encoded by pBR322), and the female plasmid recipients did not coincidentally become male (assayed by resistance to male-specific phage R17). Thus Tet^R transfers with the pBR322-derived plasmid, and the pBR322-derived plasmid has not somehow become associated with the F'. These results indicate that a replicon other than the F' is mutable during adaptive Lac reversion.

The Tet^R mutations were presumed to occur coincidentally with Lac⁺ reversion and not during growth of the Lac⁺ colonies because simple growth of a colony from a single starved or unstarved cell (on non-lactose carbon sources,

Table 3-1) was shown to be insufficient to generate high levels of Tet^R mutation. However, if the Lac⁺ revertants were heritably mutator, or if lactose selection medium provoked mutations in Lac⁺ revertants, then the comparison with colonies grown on other carbon sources would be insufficient. It has been shown that most Lac⁺ adaptive revertants do not have a heritable mutator phenotype that could accelerate unselected mutation during colony growth (Longerich *et al.*, 1995; also shown below). The unlikely alternative possibility that growth into a colony under lactose selection conditions induces the Tet^R mutations was ruled out by showing that Lac⁺ revertants isolated, replated and grown into colonies on lactose display low Tet mutation frequencies (Table 3-1). This strengthens the correlation between adaptive Lac⁺ reversion and unselected Tet^R mutation.

Sequence similarity to Lac adaptive mutation

To begin to assess whether the Tet^R mutations occur *via* a mechanism similar to that by which the F'-borne *lac* gene reverts, I examined the sequence specificity of Tet^R reversions (Figure 3-1). Unlike growth-dependent reversions which are heterogeneous, adaptive reversions of the *lac* +1 frameshift allele are nearly all -1 deletions in small mononucleotide repeats with a strong hotspot in the 4 C repeat of the original frameshift mutation (Rosenberg *et al.*, 1994; Foster and Trimarchi, 1994). This spectrum is characteristic of DNA polymerase errors that accumulate in the absence of post-synthesis mismatch repair (Longerich *et al.*, 1995). In support of a similar spectrum in the Tet^R mutations, the most active mutagenic target is a +1 frameshift allele in a run of six Gs (pW17, Table 3-1). Two other targets, a -1 G in the same G repeat, and a +GC in a GC dinucleotide repeat, are both less active (Table 3-1, Figure 3-1), in agreement with previous results regarding the sequence preference of the Lac reversion mechanism (Rosenberg *et al.*, 1994; Foster and Trimarchi, 1994). (A fourth target of unknown sequence is

also active.) Second, a region spanning the +1 G Tet mutation (Figure 3-1) was sequenced (Materials and methods) in 16 independent Tet^R reversions of the +1 frameshift Tet allele. All 16 reversions are -1 deletions in the six G repeat at position 536, the site of the original +1 frameshift mutation (Figure 3-1). These results indicate a mutation hotspot for -1 deletions in a six G repeat, consistent with the pattern seen in adaptive Lac reversion.

Mutations in the F' and the bacterial chromosome

To test whether replicons other than pBR322 experience elevated unselected mutation correlated with Lac⁺ adaptive reversion, I screened for mutations in a non-*lac* gene in the F', *codAB*, and for mutations in multiple genes in the bacterial chromosome. *codAB* encodes cytosine deaminase and transport activities, loss of function of either of which confers resistance to 5-fluorocytosine (5-FC) (Neuhard and Kelln, 1996; Lind *et al.*, 1973; De Haan *et al.*, 1972). *E. coli* can also become 5-FC-resistant (5-FC^R) by loss of function of the chromosomally located *upp* gene, which in addition confers resistance to 5-fluorouracil (5-FU) (Neuhard and Kelln, 1996; Pierard *et al.*, 1972). I obtained high frequencies of 5-FC^R and 5-FUR mutants associated with Lac⁺ adaptive reversion, but not amongst Lac⁻ stressed and unstressed cells (Table 3-2). The episomal and chromosomal location of the 5-FC^R and 5-FUR mutations respectively were confirmed by transductional mapping (Materials and methods). These results demonstrate that unselected genes on the F' and in the bacterial chromosome are mutable in association with Lac⁺ adaptive reversion. The approximate equality of 5-FUR (chromosomal) with 5-FC^R (F'-located) mutations (Table 3-2) indicates that the *upp* gene in the bacterial chromosome and the *codAB* locus in the F' are similarly mutable in association with adaptive Lac⁺ reversion.

Mutations at multiple chromosomal locations

To generalize the finding that chromosomal loci mutate in association with Lac⁺ adaptive reversion, two broad, genome-wide screens were undertaken. First, the ability to ferment the sugars xylose, maltose, and fructose was assayed by replica-plating to appropriate indicator media (Table 3-2). All three fermentation pathways are encoded by multigenic regulons and so provide large mutation targets (Böck and Sawyers, 1996; Berlyn *et al.*, 1996). The data in Table 3-2 show elevated mutation to Xyl⁻ and Mal⁻, but not Fruc⁻ phenotypes, amongst Lac⁺ adaptive revertants as compared with the two Lac⁻ control populations. All 15 Xyl⁻ and 14 Mal⁻ mutations tested were verified as being located in the bacterial chromosome (Materials and methods). Thus many, but apparently not all, chromosomal loci hypermutate in association with Lac⁺ adaptive reversion. Second, mutants temperature-sensitive (Ts) for growth on minimal medium were also enhanced amongst Lac⁺ adaptive revertants compared with Lac⁻ control populations (Table 3-2). These probably carry auxotrophic Ts mutations.

Timing of unselected mutation

I wished to address directly whether the unselected mutations associated with Lac⁺ adaptive reversion arose coincidentally with Lac⁺ reversion, and not during subsequent growth of the Lac⁺ colony. Thus, I examined the original (master) colonies from which F' and chromosomal unselected mutants were identified in the replica plating screens. The data in Table 3-3 show that most of the original 5-FC^R and 5-FUR^R Lac⁺ colonies are pure with respect to 5-FC^R and 5-FUR^R mutations, as assayed by diluting the original colonies, replating on minimal lactose medium and replica plating to 5-FC medium (on which both 5-FC^R and 5-FUR^R cells grow). 39 of 53 5-FC^R and 7 of 9 5-FUR^R mutant colonies were pure and not mixed with sensitive cells, as would have been expected if

these clones became 5-FC^R and 5-FU^R during growth of the Lac⁺ colony. This implies that most of the unselected mutations arose prior to Lac reversion while on the selective medium, coincidentally with the Lac⁺ adaptive reversion event, or prior to full expression of the Lac⁺ phenotype and subsequent cell division, and agrees with the previous finding that most Lac⁺ adaptive revertants do not have a heritable mutator phenotype (Longerich *et al.*, 1995; and below). Of the few mixed colonies obtained, most carried a few percent of 5-FCS and 5-FUS cells, and some carried a few percent of resistant cells. These probably represent independent Lac⁺ revertants that overlapped with the resistant colonies. Likewise, three of four fermentation-defective colonies were shown to be pure and not mixed with fermentation-positive cells. Therefore, the majority of unselected mutations associated with Lac reversion formed prior to Lac reversion while on the selective medium, coincidentally with Lac reversion, or prior to cell division after Lac⁺ reversion. The possibility that unselected mutations formed before Lac reversion during growth of the cultures can be excluded by their scarcity in the cultures giving rise to Lac revertants (Tables 3-1 and 3-2, Lac⁻ stressed and unstressed cells).

Mutants are not heritably mutator

As found previously for simple Lac⁺ adaptive revertants (Longerich *et al.*, 1995), most of the Lac⁺ revertants with associated unselected mutations do not possess a heritable mutator phenotype (Table 3-4). Ten each of the Tet^R, 5-FC^R, Xyl⁻, Mal⁻ mutants, nine 5-FU^R mutants, as well as all six multiple mutants isolated (Table 3-2) were analyzed for mutator phenotype as previously (Longerich *et al.*, 1995). Only 6 of the 55 possess a heritable mutator phenotype: 2 Tet^R, 1 5-FU^R, 2 Mal⁻, and 1 5-FU^R Xyl⁻ Mal⁻. Of the six that do, five have been mapped by complementation with plasmids overexpressing post-replicative mismatch repair

genes (Table 3-5). Four are complemented by a plasmid overexpressing *mutL*, and one is complemented by a plasmid overexpressing *mutS* (Table 3-5). The final heritable mutator is not complemented by plasmids overexpressing either *mutS*, or *mutL* (Table 3-5).

Discussion

The results presented imply the existence of a mutagenic process at work in the entire genome of a subpopulation of the cells exposed to selection. The mutagenic process that generates RecBC-dependent, adaptive reversions is not directed preferentially to the *lac* genes (as had been suggested by Cairns *et al.*, 1988; Hall, 1988; Boe, 1990; Davis, 1989; Cairns and Foster, 1991; Foster, 1993, 1994), to a region of DNA around the *lac* genes (as was implied in models of Foster, 1993; Roth *et al.*, 1996), to the F' replicon on which the *lac* genes reside (as was predicted by Radicella *et al.*, 1995; Galitski and Roth, 1995; Foster and Trimarchi, 1995; Peters and Benson, 1995; Foster *et al.*, 1996), or to episomes in general. Multiple loci in multiple *E. coli* replicons, including the bacterial chromosome, are hypermutated in association with Lac⁺ adaptive reversion.

An alternative interpretation is that a second, correlated but different mutagenic mechanism also operates in cells that mutate adaptively. This is a more complicated hypothesis and is argued against by the similarity of the sequences of the Tet^R adaptive reversion-associated mutations to Lac⁺ adaptive reversions (Rosenberg *et al.*, 1994; Foster and Trimarchi, 1994).

A hypermutable subpopulation

Hypermutation of unselected genes is seen amongst adaptive Lac⁺ revertants but not amongst Lac⁻ cells from the same starved cultures. This implies that a subpopulation of cells exposed to starvation on lactose experiences a genome-wide hypermutable state (Rosenberg, 1994; Rosenberg *et al.*, 1995, 1996). This state must be transient because most adaptive revertants are not heritably mutator (Longerich *et al.*, 1995; Table IV). Such a hypermutable subpopulation could be induced by selection, stationary phase, or stress (Rosenberg, 1994; Rosenberg *et al.*, 1995, 1996).

A similar conclusion, based on fewer data, was inferred for Trp⁺ adaptive reversion, which occurs *via* a different, recombination gene-independent mechanism (Hall, 1990, 1995). Hall suggested a selection-induced hypermutable state in which a small subpopulation of the cells experiences genome-wide mutagenesis, but ultimately dies unless a selected (adaptive) mutation is generated (Hall, 1990). This type of model (see also Ninio, 1991; Harris *et al.*, 1994) is supported for RecBC-dependent Lac⁺ reversion by the data presented, and a molecular basis of such death has been suggested previously (Harris *et al.*, 1994; Rosenberg 1994; Rosenberg *et al.* 1995, 1996). However, the data presented here do not require invoking death: Lac⁺ revertants represent about 10⁻⁶ of the population (*e.g.* see Harris *et al.*, 1994, 1996; also true here). If unselected mutations occurred in 10⁻⁶ Lac⁻ starved cells they would not have been detected by our screen. Previous failure to detect unselected mutations in the whole population might be because the only unselected mutation target used could not detect frameshift mutations (Foster, 1994).

The size of the subpopulation that gives rise to adaptive Lac revertants can be estimated as follows: If the mutations in unselected genes occurred randomly, then the numbers of single (Lac⁺) mutants, double, and triple mutants should fall

within a Poisson distribution. The data for single, double, and triple mutants from a single large experiment reported in Table 3-2 conform to a Poisson distribution with a mutation rate of 0.5×10^{-2} per day in about 10^{-5} of the whole population (see appendixD for calculations). The implication of being able to fit these data to a Poisson distribution is that the mutation rates for unselected genes are similar to that for *lac*.

Additional evolutionary implications

Subpopulations of mutant cells have been found to arise and then overtake stationary-phase bacterial cultures (Zambrano and Kolter, 1996). These so-called GASP mutants (Growth Advantage in Stationary Phase) appear to provide variation that allows evolution during the harsh condition of stationary phase. A hypermutable subpopulation of stationary-phase cells such as that reported here could lead to the formation of GASP mutants. The hypermutation could be part of a developmental program for generating subpopulations with GASP ability. This idea, that the environment influences mutation rate, contrasts with the neo-Darwinian tenet of a uniform mutation rate. However, Darwin's formulation of natural selection included this idea (Darwin, 1859).

Molecular mechanism of recombination-dependent stationary-phase mutation

A current list of aspects of the molecular mechanism of recombination-dependent mutation in this system follows:

- (1) The process of Lac adaptive reversion includes a requirement for recombination genes of the RecBCD system (Harris *et al.*, 1994, 1996; Foster *et al.*, 1996).

(2) RecBCD involvement implicates double-strand DNA breaks (DSBs) as a molecular intermediate because RecBCD loads onto DNA only at DSBs (Taylor, 1988; Kowalczykowski *et al.*, 1994; Myers and Stahl, 1994).

(3) The adaptive mutation sequences resemble DNA polymerase errors (Rosenberg *et al.*, 1994; Foster and Trimarchi, 1994), which are probably made by DNA polymerase III (Foster *et al.*, 1995; Harris *et al.*, 1997a).

(4) The polymerase errors resemble those that accumulate in the absence of mismatch repair (Longerich *et al.*, 1995). Mismatch repair appears to be diminished during adaptive mutation by a transient deficiency in functional mismatch repair protein (Harris *et al.*, 1997b).

(5) F-plasmid transfer (Tra) genes are required for Rec-dependent adaptive reversion of *lac* on the F', and two non-F' sites tested do not produce Rec-dependent Lac reversions (Radicella *et al.*, 1995; Galitski and Roth, 1995; Foster and Trimarchi, 1995; R. S. Harris and S. M. Rosenberg, unpublished results). However, other loci in other replicons are hypermutable during adaptive mutagenesis (this chapter).

One way to assemble these pieces follows (see Rosenberg, 1994; Rosenberg *et al.*, 1995, 1996): DSBs generated in a subpopulation of the stressed cells would promote high levels of RecABCD-mediated recombination (Harris *et al.*, 1994). DNA synthesis associated with recombination could include polymerase errors (Harris *et al.*, 1994) which persist as mutations due to down-regulation of mismatch repair (Rosenberg *et al.*, 1994; Foster and Trimarchi, 1994; Longerich *et al.*, 1995; Harris *et al.*, 1997b).

What DNA recombines?

The finding of unselected mutation in replicons other than the F' bears on the question: "With what DNA does the double-strand-broken DNA recombine?"

The DNA homology used for recombination in recombination-dependent mutation could have been either a whole or partial sister replicon (Harris *et al.*, 1994), a gene duplication (amplification) (Harris *et al.*, 1994), or exogenous DNA taken-up into the starving cells. However, if duplications were the homology source, then unselected mutations associated with adaptive reversions might have been confined to sites in the duplicated DNA segment, *i.e.* next to the adaptive reversion. Thus this gene duplication hypothesis and others (Foster, 1993; Roth *et al.*, 1996) appear less tenable in light of finding genome-wide hypermutation.

Role of F transfer proteins

The role of F transfer proteins may be single-strand nicking of the F transfer origin by the Tra proteins. This nick could be converted to a DSB by, for example, endonuclease activity (Rosenberg *et al.*, 1995), by replication (Harris *et al.*, 1994; Kuzminov, 1995; Foster *et al.*, 1996; Rosenberg *et al.*, 1996), or by DNA repair-like single-strand excision and/or synthesis up to the nick on the opposite strand. Chromosomal and pBR322-located mutable sites could experience DSBs similarly, by two opposite single-strand nicks (but with neither caused by Tra; Rosenberg *et al.*, 1995), or by other mechanisms (Harris *et al.*, 1994; Rosenberg *et al.*, 1996). There is evidence that different chromosomal regions are differently susceptible to DSBs (reviewed by Rosenberg *et al.*, 1995, 1996). This could account for the existence of two sites in the bacterial chromosome that are inactive for recombination-dependent Lac reversion (Radicella *et al.*, 1995; Galitski and Roth, 1995; Foster and Trimarchi, 1995; R. S. Harris and S. M. Rosenberg, unpublished results), and does not exclude the possibility that other chromosomal sites will be active.

Models invoking transfer, transfer synthesis, or sex as a precondition to the mutagenesis in this system predict that unselected mutation in association with Lac adaptive reversion would be confined to the F' (Galitski and Roth, 1995; Radicella *et al.*, 1995; Foster and Trimarchi, 1995; Peters and Benson, 1995; Rosenberg *et al.*, 1995). This prediction appears to be inconsistent with the results reported here.

Generality of the mechanism

The discovery of genome-wide hypermutability underlying this novel recombination-dependent mutagenic mechanism in stressed, non-growing cells re-opens the possibility that this mechanism may apply more broadly. Associations between recombination and either mutations or DNA synthesis have been inferred in other systems. These include mutations associated with double-strand break-repair in yeast, (Strathern *et al.*, 1995), mutagenesis correlated with recombination, sex, or both in bacteria (Demerec, 1962, 1963), yeast (Magni and von Borstel, 1962; Esposito and Bruschi, 1993), and filamentous fungi (Paszewski and Surzycki, 1964), and the association of hyper-recombination or chromosome instability with elevated mutation in Bloom's and Werner's syndromes in humans (reviewed by German, 1993; Yu *et al.*, 1996). DNA synthesis promoted by recombination is thought to be the source of replication origin-independent "inducible stable DNA replication" (iSDR) in *E. coli* which has similar but not identical genetic requirements to Rec-dependent Lac reversion (Asai and Kogoma, 1994a; 1994b; Foster *et al.*, 1996; Harris *et al.*, 1996).

Thus, the mutagenic mechanism being elucidated in the *lac* system could be relevant in microbial evolution and in other organisms, during normal

development and, for example, in the origin of cancer. The abundance of *E. coli* recombination and mutator protein homologs already implicated in such processes (Modrich, 1994; Ellis *et al.*, 1995; Yu *et al.*, 1996) underscores the need to understand all of the ways that these proteins promote genetic change in *E. coli* and in more complicated organisms whose proteins are structurally and functionally similar.

Materials and methods

Obtaining adaptive mutants, Lac⁻ stressed and unstressed cell colonies

Lac⁺ adaptive revertants of the *lacI*-Z fusion *lacI* frameshift-bearing strain were obtained after 3-7 days incubation on M9 thiamine 0.1% lactose medium (Cairns and Foster, 1991; Harris *et al.*, 1994, 1996) (+ 100µg/ml ampicillin for experiments in Table 3-1). Day 3-7 colonies were marked on the backs of the plates and replica-plated. Lac⁻ stressed colonies were obtained by resuspending agar taken from between visible Lac⁺ adaptive mutant colonies on days 4-6, in M9 salts, and plating on LBH rifampicin plates or on M9 thiamine 0.2% glucose rifampicin plates (LBH, 1% tryptone, 0.5% NaCl, 0.5% yeast extract, 2µg/ml thymine, pH 7, plate media solidified with 1.5% agar, rifampicin, 100µg/ml + 100µg/ml ampicillin for experiments in Table 3-1) on which the *lac⁻* frameshift-bearing cells form colonies, but the *lac*-deleted scavenger cells plated with them do not. Scavenger cells consume any non-lactose carbon sources (Cairns and Foster, 1991). Lac⁻ unstressed colonies were grown on M9 thiamine 0.1% glycerol plates (+ 100µg/ml ampicillin for experiments in Table I).

Sequencing

Tet^R plasmids were isolated, transformed into plasmid-free cells and Tet^R selected to purify the dominant mutant plasmid from Tet^S parental plasmids prior to sequencing. Template DNA prepared by alkaline lysis was purified in QIAprep spin columns (Qiagen). An oligonucleotide primer complementary to the sequence "Primer complement" in Figure 3-1 (synthesized on an ABI Model 392 Synthesizer, Applied Biosystems, Foster City, CA) was used for double-stranded sequencing of DNA from the region shown (see Rosenberg *et al.*, 1994).

Identification of unselected mutations

Tet^R mutants were identified on LBH + 20µg/ml tetracycline plates. 5-FC^R mutants were identified on M9 thiamine glycerol or lactose with 20µg/ml 5-FC. Those also 5-FU^R were identified by spotting saturated cultures derived from isolated 5-FC^R colonies onto M9 thiamine glycerol or lactose plates with 10µg/ml 5-FU. Mutants defective in fermentation of xylose (Xyl⁻), maltose (Mal⁻), and fructose (Fruc⁻) were identified as white or pink colonies on MacConkey indicator solid medium (Difco) containing 1% of these sugars and rifampicin. The Xyl⁻ and Mal⁻ were then confirmed on minimal xylose and maltose media. Mutants temperature-sensitive (Ts) for growth on minimal glycerol medium but not on rich medium, were identified by replica-plating the two Lac⁻ control colony populations to M9 thiamine 0.1% glycerol plates, and to rich LBH plates. Both were incubated at 37° and 43°. Those that grow at 37° but not 43° only on minimal medium are designated Minimal Ts, and are probably auxotrophic-Ts mutants. (General Ts mutants were too few to quantify in this assay.) None is Ts for proline biosynthesis (all are ts on minimal glycerol proline plates), the only amino acid biosynthetic locus on the F'. For the Lac⁺ revertants, phenotypic Lac⁻ Ts colonies (presumed *lac*-Ts frameshift mutants), which represent a few percent

of the total, were screened for other Ts by replica-plating to minimal lactose medium at 37° and 43°. Those that failed to grow at 43° were diluted serially by consecutive toothpicking in minimal lactose rifampicin agar (to purify Lac⁺ revertants away from non-revertant and rifampicin-sensitive scavenger cells; Cairns and Foster, 1991), incubated at 37°, then replica-plated to minimal glycerol plates at 37° and 43°. All Minimal Ts mutants isolated were confirmed by picking the original Lac⁺ colony, diluting and plating for single colonies on minimal lactose rifampicin at 37° then replica-plating to minimal glycerol at 37° and 43°.

Mapping unselected mutations

5-FCR^R and 5-FUR^R mutations were mapped to the F' and the chromosome by P1 transductional mapping. The *codAB* locus, whose loss of function confers 5-FCR^R, is located next to *lac* on the F' and is deleted from the bacterial chromosome in this strain (Cairns and Foster, 1991). Phage P1 transductional mapping demonstrated linkage of all 49 independent 5-FCR^R mutations tested to the *lac* gene. The *lac*⁻ *codA*⁺ strain BW7620 (*E. coli* Genetic Stock Center number CGSC6813) was transduced with P1 grown on each 5-FCR^R isolate, Lac⁺ colonies selected, and co-transduction of 5-FCR^R mutant phenotype observed. The *upp* gene, whose loss of function confers both 5-FCR^R and 5-FUR^R, is located next to *guaBA* in the bacterial chromosome and is not present on the F'. P1 transductional mapping showed linkage of all 35 independent 5-FUR^R mutations tested to the deletion $\Delta(gua-xseA)$ (Vales *et al.*, 1979). Guanosine-proficient transductants of a $\Delta(gua-xseA)$ strain were 5-FUR^R at the expected frequency. 15 Xyl⁻ and 14 Mal⁻ mutations tested were confirmed as chromosomal by the ability of the mutants to be transduced to Xyl⁺ and Mal⁺ phenotypes with phage P1 grown on strain P90C (Cairns and Foster, 1991), the female parent of the *lac*

frameshift-bearing strain. P90C carries no F' and has an identical chromosomal *lac* deletion as in the frameshift-bearing strain.

Note added in proof: A paper published after this manuscript's acceptance could seem to suggest that unselected mutation during Lac adaptive mutation is confined to the F' [PL Foster. (1997). Nonadaptive mutations occur on the F' episome during adaptive mutation conditions in *Escherichia coli*. *J. Bacteriol.* 179, 1550-1554.]. However, as no non-F' sites were examined, the data are compatible with those reported here.

Table 3-1. Plasmid-borne Tet^R mutations are associated with Lac⁺ adaptive reversion.

Plasmid name ^a	Tet ^S mutation	Tet ^S mutation nucleotide position ^b	Number of Tet ^R mutants among Lac ⁺ adaptive revertants (Tet ^R colonies / colonies scored)	Number of Tet ^R mutants among Lac ⁻ stressed cells (Tet ^R colonies / colonies scored)	Number of Tet ^R mutants among Lac ⁻ unstressed cells (Tet ^R colonies / colonies scored)	Number of Tet ^R mutants among Lac ⁺ adaptive revertants grown on lactose ^c (Tet ^R colonies / colonies scored)
pW17	+1 G	536	93 / 27,784	0 / 8894	3 / 26,097	0 / 14,513
pW18	-1 G	536	0 / 2329	0 / 5687	0 / 8578	nt ^d
pX2	+GC	435	0 / 3626	0 / 4407	0 / 12,554	nt ^d
pRDK35	oligo-nucleotide insertion	23	12 / 1684	0 / 4176	nt ^d	nt ^d

Mutation to tetracycline-resistance (Tet^R) is dominant and the plasmids are multicopy, such that one Tet^R mutation among the multiple Tet gene targets will produce Tet^R, thus providing a sensitive assay.

^aDerivatives of the *lac* frameshift-bearing strain (Cairns and Foster, 1991) carry pW17, pW18 (Koffel-Schwartz et al., 1984), pX2 (Burnhouf and Fuchs, 1985), or pRDK35 (Doherty et al., 1983), all of which are identical to pBR322 (Sutcliffe, 1979) except for frameshift mutations in the tetracycline resistance (Tet) gene (see Figure 1).

^bSee Figure 3-1 for sequence position and context information.

^cThis control shows that growth of a Lac⁺ cell into a colony under lactose selection conditions is not sufficient to produce associated Tet^R mutations.

^dnt, not tested.

332
GCCACTATCGACTACGCGATCATGGCGACCACACCCGTCCTGTGGATCCT

CTACGCCGGACGCATCGTGGCCGGCATCACCGGCGCCACAGGTGCGGTT

435
GCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGATCGGGCTCG

CCACTTCGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCC

536
CCGTGGCCGGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATT

CCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCC

624
TAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTTGAGAGC

CTTCAACCCAGTCAGCTC
< Primer complement

Figure 3-1. DNA Sequence of a Region of the Tetracycline Resistance (Tet) Gene of Plasmid pW17. pW17 carries a +1 G mutation at position 536 abolishing function of the Tet gene (Koffel-Schwartz *et al.*, 1984). Tet⁺ mutations could restore the reading frame by addition or deletion between the two stop codons spanning the mutation. The downstream, +1 stop codon is shown (italics). The upstream -1 stop codon occurs in a region upstream of the sequenced region shown. Nucleotide position numbers apply to the left-most base immediately below them, and correspond to those used for pBR322 (Sutcliffe, 1979) with the +1 G present in pW17 unnumbered.

Table 3-2. F and chromosomal unselected mutations are associated with Lac⁺ adaptive reversion.

Replicon carrying the unselected mutation	Mutant phenotype ^a	Expt.	Number of mutants among Lac ⁺ adaptive revertants (mutant colonies / colonies scored)	Number of mutants among Lac ⁻ stressed cells (mutant colonies / colonies scored)	Number of mutants among Lac ⁻ unstressed cells (mutant colonies / colonies scored)
F'	5-FCR	1	121 / 19,647	1 / 17,876	2 / 21,940
	5-FCR	2	115 / 41,238	0 / 66,274	0 / 12,860
Chromosome	5-FUR	1	nt ^b	nt ^b	nt ^b
	5-FUR	2	121 / 41,238	1 / 66,274	1 / 12,860
	Xyl ^{-c}		22 / 42,617	3 / 69,795	0 / 26,025
	Mal ^{-c}		31 / 42,617	0 / 69,795	0 / 23,471
	Fruc ^{-c}		2 / 42,617	1 / 69,795	1 / 37,961
	Minimal Ts		10 / 44,984	1 / 71,709	1 / 27,265

^aA few of the mutants listed among Lac⁺ are multiple: two 5-FC^R Mal⁻; one 5-FU^R Mal⁻; one 5-FU^R Xyl⁻ Mal⁻; one Xyl⁻ Mal⁻; one Mal⁻ Minimal Ts. Transductional analyses indicate that all of these are separately heritable and thus represent different mutations.

^bnt, not tested.

^cThe numbers of fermentation mutants amongst Lac⁺ revertants are underestimates because the Lac⁻ background forms a film of fermentation-positive (red) growth after replica plating. This is not the case for the Lac⁻ control populations. The underestimation may be most serious for Fruc⁻ in which only pink (no white) mutants were found.

Table 3-3. Lac⁺ revertant colonies carrying associated unselected mutations are mostly pure clones^a.

Unselected mutant ^b	Number of Lac ⁺ master colonies tested ^c	Phenotyped ^d
5-FCR	39	pure
	14	mixed
5-FUR	7	pure
	2	mixed
Mal ⁻	2	pure
	1	mixed
Xyl ⁻	1	pure
Total	66	49 pure 17 mixed

^aIf the original Lac⁺ master colony is pure such that all cells in that colony carry the unselected mutation, then the unselected mutation could not have arisen subsequent to Lac⁺, during growth of the Lac⁺ cell into a colony.

^bF⁻ and chromosomal unselected mutants were analyzed whereas pBR322-borne Tet^R mutants were not because Tet^R is a dominant mutation carried on a multicopy replicon. Therefore, all Lac⁺ master colonies with Tet^R mutations should be mixed with respect to the number of cells per colony carrying the Tet^R mutation, even if the Tet^R mutation formed coincidentally with Lac⁺.

^cIn order to avoid analyzing colonies contaminated by other nearby colonies, only master colonies that were separated from other colonies on the plate by at

least 0.5 cm were examined. This necessitated exclusion of all of the fermentation mutants other than the four reported.

^dThe original Lac⁺ master colonies whose replica plating led to identification of unselected mutants were picked, suspended and replated to minimal lactose medium. The colonies that arose were then replica plated to 5-FC medium for both 5-FC^R and 5-FU^R, and to minimal maltose and minimal xylose media for Mal⁻ and Xyl⁻, respectively. Between 40-660 individual colonies were examined per Lac⁺ master colony for all except six of the 5-FC^R master colonies in which the numbers tested were 5, 14, 20, 22, 36 and 36. "Pure" master colonies are those that showed zero derived colonies not carrying the unselected mutation. The percent of non-mutants per mixed master colony ranged from 1 to 100 (of the derived colonies examined per master colony) and was distributed such that 13 of the 17 mixed colonies contained mostly one phenotype with only a small percentage of the other, whereas 4 contained between 25-51% non-mutant. All of these may represent overlapping Lac⁺ master colonies, however the latter four might also be derived by segregation of a heterozygous unselected mutation that arose close to the same time as Lac⁺.

Table 3-4. Most unselected mutants associated with Lac reversion are not heritably mutator.

Strain ^a	Number of independent isolates tested	Number of independent cultures tested per isolate	Number of spots tested per culture	Mutant colonies per spot ^b [Mean ($\pm$ SE)] ^c		Phenotype
				Nalidixic acid ^R	Streptomycin ^R	
Lac ⁻ parent	1	4	25	0.05 ($\pm$ 0.006)	0.01 ($\pm$ 0.002)	Non-Mutator
Lac ⁻ parent[pW17]	1	4	25	0.03 ($\pm$ 0.004)	<0.01	Non-Mutator
Lac ⁻ mutL	1	4	10	>25 ($\pm$ 1) ^d	1.1 ($\pm$ 0.1)	Mutator
Lac ⁻ dam	1	4	10	4.1 ($\pm$ 0.2)	0.05 ($\pm$ 0.02)	Mutator
Lac ⁺ Tet ^R	8	4	1	<0.03	<0.03	8 Non-Mutator
Lac ⁺ Tet ^R	1	4	1	15 ($\pm$ 7)	<0.03	Mutator
Lac ⁺ Tet ^R	1	4	1	11 ($\pm$ 3)	<0.03	Mutator
Lac ⁺ 5-FC ^R	10	4	1	0.075 ($\pm$ 0.02)	0.025 ($\pm$ 0.01)	10 Non-Mutator
Lac ⁺ 5-FUR	8	4	1	<0.03	0.03 ($\pm$ 0.02)	8 Non-Mutator
Lac ⁺ 5-FUR	1	4	1	18 ($\pm$ 2)	2 ($\pm$ 0.7)	Mutator
Lac ⁺ Mal ⁻	8	4	1	<0.03	<0.03	8 Non-Mutator

Table 3-4 continued...

Lac ⁺ Mal ⁻	1	4	1	26 (±5)	1.5 (±0.5)	Mutator
Lac ⁺ Mal ⁻	1	4	1	6.3 (±1)	0.75 (±0.3)	Mutator
Lac ⁺ Xyl ⁻	10	4	1	0.075 (±0.08)	0.075 (±0.04)	10 Non-Mutator
Lac ⁺ 5-FCR Mal ⁻	2	4	1	0.12 (±0.1)	<0.1	2 Non-Mutator
Lac ⁺ 5-FUR Mal ⁻	1	4	1	<0.3	<0.3	Non-Mutator
Lac ⁺ Mal ⁻ Xyl ⁻	1	4	1	<0.3	<0.3	Non-Mutator
Lac ⁺ Mal ⁻ Ts	1	4	1	<0.3	<0.3	Non-Mutator
Lac ⁺ 5-FUR Mal ⁻ Xyl ⁻	1	4	1	7.0 (±2)	0.6 (±0.3)	Mutator
Totals	55					49 Non-Mutator 6 Mutator

^aAll are isogenic derivatives of the *lac* frameshift-bearing strain (Cairns and Foster, 1991) designated Lac⁻ parent here. The *mutL* and *dam* derivatives (Longerich *et al.*, 1995) are positive controls for strong and weak mutator phenotypes, respectively. Isolation of the Lac⁺ revertants with associated unselected mutations is described in Tables I and II.

^bSpots of 10μl of culture grown to saturation were made onto solid media containing 100μg/ml streptomycin or 40μg/ml nalidixic acid, the plates incubated and the number of mutant colonies per spot counted.

^cSE, one standard error of the mean.

^dGreater than 50 colonies per spot causes confluent growth and is uncountable. The number of colonies recorded for such uncountable spots was >50. The calculations were made based on 50 and the final values marked >.

Table 3-5. Mutator complementation analysis

Strain / plasmid ^d	Average Number of Naladixic Acid Resistant Colonies ^a [Mean($\pm$ SE)] ^b				Average Number of Streptomycin Resistant Colonies [Mean($\pm$ SE)]			
	none	pcontrol	pmutS	pmutL	none	pcontrol	pmutS	pmutL
77	19 (1.6)	14 (1.9)	3 (2.6)	0.10 (0.10)	0.80 (0.50)	1.2 (0.60)	2.5 (1.6)	<0.10
84	17 (2.9)	12 (2.4)	10 (2.6)	<0.10	1.6 (0.70)	3.7 (2.3)	1.9 (1.2)	0.10 (0.10)
424	12 (1.4)	20 (3.2)	19 (3.7)	0.70 (0.60)	1.2 (0.70)	0.20 (0.16)	0.50 (0.40)	<0.10
470	6.8 (1.3)	9.4 (2.1)	<0.10	7.5 (1.4)	1.2 (0.70)	1.0 (0.30)	<0.10	0.70 (0.20)
513	14 (2.3)	10 (2.0)	25 (7.0)	<0.10	1.0 (0.50)	0.80 (0.30)	1.0 (0.50)	0.10 (0.10)
528	11 (2.0)	4.1 (0.90)	3.2 (0.90)	1.9 (0.50)	0.20 (0.20)	0.30 (0.30)	1.3 (1.1)	0.20 (0.10)
<i>mutS</i>	31 (5.4)	nd	<0.20	nd	4.8 (1.9)	nd	<0.20	nd
<i>mutL</i>	34 (4.8)	nd	nd	<0.20	2.6 (0.40)	nd	nd	<0.20
FC40	<0.20	nd	nd	nd	<0.20	nd	nd	nd

^a Saturated cultures of all strains were made in LBH broth and 50 μ g/ml kanamycin, for those bearing plasmids, and incubated at 37°C. Ten 10 μ l aliquots from each saturated culture of the mutators with and without plasmids were spotted onto either LBH 40 μ g/ml naladixic acid or 100 μ g/ml streptomycin

media, and five 10 μ l aliquots of the *mutS*, *mutL*, and FC40 strains with and without plasmids were spotted onto the same media. Plates were incubated overnight at 37°C and the spots were scored for colony growth.

^b SE, one standard error of the mean.

^c All strains are in the frameshift-bearing FC40 background. Strain numbers refer to unselected mutant collection and phenotypes are described in appendix B. Mutator strains were transformed with plasmids overexpressing post-replicative mismatch repair genes by CaCl₂ transformation. FC40*mutS*::Tn10, FC40*mutL*::Tn10, FC40*mutS*::Tn10pSL6, and FC40*mutL*::Tn10pSL5 are SMR749, SMR1712, SMR1748, and SMR1746, respectively.

^d Plasmids overexpressing *mutS*, *mutL*, were pSL6 and pSL5, respectively. The control plasmid was pSL4.
nd, no data.

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Chapter 4
Conclusions and Discussion

Summary

The results described in this thesis answer 3 controversial and important questions about adaptive mutation. First, using a replica plating screen for unselected mutations, I found that adaptive mutation, in this system, is not directed in a Lamarckian manner exclusively to the gene under selection (chapter 3; Torkelson *et al.*, 1997; Foster, 1997). The observation of a high frequency of mutation in all replicons in the cell associated with Lac⁺ adaptive reversion lends support to a random Darwinian mechanism for the generation of adaptive mutations (chapter 3; Torkelson *et al.*, 1997).

The sequence spectrum of adaptive Lac⁺ revertants is largely -1 deletions in small mononucleotide repeats (Foster and Trimarchi, 1994; Rosenberg, *et al.*, 1994). In support of a similar mechanism of formation of mutation, the sequence spectrum of Tet^R unselected mutations on a pBR322-derived plasmid was also exclusively -1 deletions in a mononucleotide repeat (chapter 3; Torkelson *et al.*, 1997).

Multiple chromosomal loci displayed hypermutation in association with Lac⁺ adaptive reversion (chapter 3; see also Torkelson *et al.*, 1997). In fact, one chromosomal locus, *upp*, displayed an approximately equal frequency of mutation as did the *codAB* locus on the F'-episome (chapter 3; Torkelson *et al.*, 1997). This suggests that the chromosome is just as accessible to this mutational process as is the F'. Two loci on the chromosome, however, have been shown to be inactive for recombination-dependent Lac⁺ reversion (Foster and Trimarchi, 1995a; Galitski and Roth, 1995; Radicella, *et al.*, 1995). The results presented in this thesis are consistent with the former, as hypermutation to a Fruc⁻ phenotype was not observed (chapter 3; Torkelson *et al.*, 1997). Perhaps, some but not all chromosomal loci may be accessible to this mutational mechanism. The

determining factor might be whether the locus is in a region that experiences high levels of DSBs during stationary-phase (Rosenberg, *et al.*, 1996; Rosenberg, *et al.*, 1995).

Those Lac⁺ stationary-phase revertants that carry unselected mutations could be the result of a mutation that confers a mutator phenotype. However, the majority of those tested do not possess a heritable mutator phenotype (chapter 3; Torkelson *et al.*, 1997). This supports the existence of a transient hypermutable state.

Second, the results presented demonstrate that stationary-phase mutations are not confined exclusively to the F-episome (chapter 2; chapter 3; Rosenberg *et al.*, 1995; Torkelson *et al.*, 1997). The finding of unselected mutations on non-F replicons (chapter 3; Torkelson *et al.*, 1997) and the finding that the *rec*-genotype of the scavenger has no effect on mutation rate (chapter 2; Rosenberg *et al.*, 1995) discourages transfer synthesis models for the generation of stationary-phase mutations (Foster, 1997; Foster and Trimarchi, 1995a; Foster and Trimarchi, 1995b; Galitski and Roth, 1995; Peters and Benson, 1995; Radicella, *et al.*, 1995).

Third, hypermutation in unselected genes was not detected amongst Lac⁻ cells under identical starvation conditions as Lac⁺ revertants (chapter 3; Torkelson *et al.*, 1997). This suggests that a subpopulation of cells exposed to selection experiences a genome-wide hypermutable state (Hall, 1990; Rosenberg, 1994; Rosenberg, *et al.*, 1996; Rosenberg, *et al.*, 1995). This hypermutable state must be transient as most adaptive revertants are not heritably mutator (Longerich, *et al.*, 1995; chapter 3; Torkelson *et al.*, 1997). This hypermutable state in a subpopulation of cells may be a regulated process and could be induced by selection, stationary-phase, or starvation (Rosenberg, 1994; Rosenberg, *et al.*, 1996; Rosenberg, *et al.*, 1995). Alternatively, the subpopulation might exist before exposure to selective conditions. Jeffrey Miller has suggested that cells

under starvation conditions may simply be falling apart and scavenging their proteins, among the first of which to go may be mismatch repair proteins (Hopkin, 1997). This is argued against by the abundance of MutL in stationary-phase (Harris *et al.*, 1997). The protein is present but inactive. It has not been scavenged.

Models for stationary-phase mutation

These results along with the molecular handles on adaptive mutation allows one to formulate a model (Rosenberg, 1994; Rosenberg, *et al.*, 1996; Rosenberg, *et al.*, 1995). A subpopulation of cells under stress due to starvation generates the formation of DSBs, which could promote high levels of RecABCD-mediated homologous recombination (Harris, *et al.*, 1994). Errors associated with DNA synthesis primed by recombination intermediates (such as 3' single stranded DNA) (Harris, *et al.*, 1994) could persist as mutations due to a transient decrease in functional mismatch repair (Foster and Trimarchi, 1994; Harris, *et al.*, 1997; Longerich *et al.*, 1995; Rosenberg, *et al.*, 1994). If the mutation is to Lac⁺, the cell could exit the hypermutable state, stop making DSBs, and live to form a colony. If the mutation is not to Lac⁺, the cell continues making DSBs and perhaps dies as a result.

Do we need death in our model? Results of Foster (1997) suggest that we do not. It has been shown that Lac⁻ cells when under selection to become Lac⁺, accumulate mutations in a second mutational target on the F-episome (Foster, 1997). Foster (1997) constructed a strain with a defective Tn10 element on the F-episome along with the *lacI33 Ω lacZ* allele, such that it no longer encodes tetracycline resistance (Tet^R). Foster (1997) found that Tet^R mutations arise at about 80% the rate of mutations to Lac⁺ amongst Lac⁻ cells under selection to

become Lac⁺. This implies that the subpopulation of cells undergoing the hypermutable state (chapter 3; Torkelson *et al.*, 1997) do not die when they do not generate a Lac⁺ mutation. Are these results inconsistent with those presented in this thesis? No, they are not. First, our replica plating screen would not be able to detect unselected mutations that occur in only 10⁻⁶ of the Lac⁻ cells under starvation (chapter 3; Torkelson *et al.*, 1997) as Foster's (1997) unselected mutations did. If generally true, the results of Foster (1997) indicate that cells undergoing recombination-dependent mutation do not die if they fail to generate a selected mutation. We nevertheless demonstrate a hypermutable subpopulation that produces recombination-dependent stationary-phase mutations. Thus, the model in Figure 4-1 includes a hypermutable subpopulation, but unlike Hall's model (1990), does not include death of the hypermutable cells if an adaptive mutation is not formed.

This kind of stationary-phase mutation or mutation in non-growing cells could have broad implications for bacterial evolution and the generation of mutations in non-dividing (and dividing) somatic cells in mammals. So although adaptive mutation in this system is not directed, the discovery of a unique pathway to the formation of mutation provides a fundamental addition to our understanding of mutational variation. It should also be recognized that the idea of directed mutation for other systems and organisms has not been disproven.

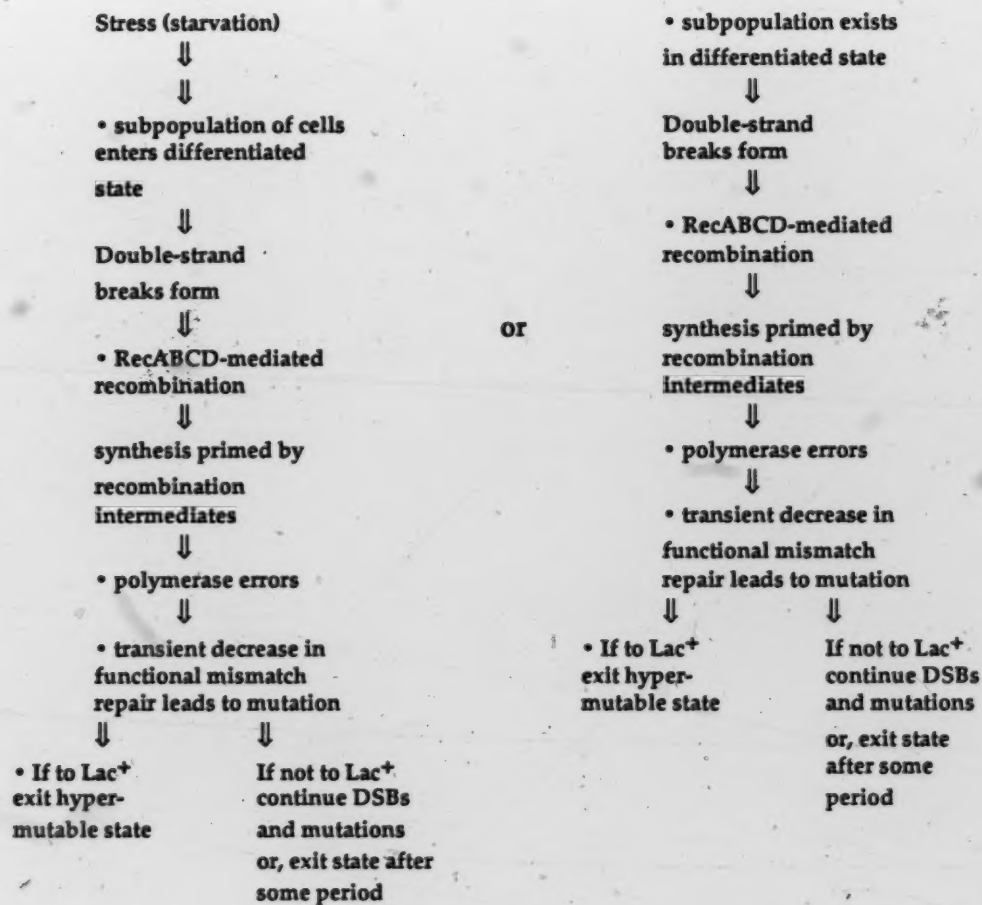


Figure 4-1. Models for the generation of stationary-phase mutations

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Appendix A

Determination of the state of the F' in FC40:

Is Hfr formation correlated with adaptive mutation?

Introduction

The finding of unselected mutation on the chromosome in association with Lac⁺ adaptive reversion begs the question of how these unselected mutations arose (chapter 3; see also Torkelson *et al.*, 1997). Our model predicts that DSBs promote the formation of RecBCD-mediated homologous recombination intermediates which prime synthesis and this ultimately generates a mutation. However, for unselected mutations in the chromosome, the possibility exists that the F' integrates into the chromosome forming an Hfr strain. If this recombination process were imprecise, integration could result in a chromosomal mutation, perhaps by interruption of an open reading frame. This could occur in the entire population of cells starving under minimal lactose selection or alternatively, occur only in the reverting subpopulation of frameshift-bearing cells. Another possibility, is that the entire population of cells, or again just the subpopulation, pre-exists as an Hfr strain, and DSBs forming as a result of nicking at *oriT* could generate Lac⁺ adaptive mutations and/or unselected mutations associated with Lac⁺ reversion. If this were the case, we would expect that only some mutations could be formed by this process, as the F' does not integrate randomly into the chromosome (reviewed by Low, 1996). This would not explain the generation of unselected mutation on pBR322-derived plasmids.

We can begin to address these possibilities by determining the state of the F' in our original Lac⁻ isolate of FC40, as well as in a number of Lac⁺ revertants and in a number of chromosomal unselected mutants. To do this I performed mating experiments using, the above mentioned populations, as donors, and AB1157 as a recipient. The strain AB1157 is auxotrophic for a number of amino acids as well as being defective in fermentation of a

number of sugars. It is useful as it provides selections that encompass loci in different regions all around the *E. coli* chromosome. To differentiate between F' and Hfr two different tests were made. First, matings were interrupted at 20 minutes and at 200 minutes. At 20 minutes, only those cells that are F' strains should be able to transfer their F' to the recipient, forming a transconjugant. At 200 minutes both F' and Hfr strains should be able to transfer their DNA to the recipient strain. Second, the matings were performed in parallel with both a Rec⁺ and a Rec⁻ recipient. The *rec* genotype of the recipient has no effect on transfer from an F' donor (Clark & Margulies, 1965). Alternatively, the subsequent recombination of transferred DNA from an Hfr cell requires a functional *recA* gene in the recipient (Cullum and Broda, 1979).

Results and Discussion

Matings were performed in liquid medium with the recipient in excess. Figure A-1 displays the locations on the *E. coli* chromosome of loci in the maltose regulon, the xylose regulon, the locus *upp*, mutant loci of AB1157, and the insertion points of all Hfr strains used in mating experiments. The data in Table A-1 show that FC40, Lac⁺ revertants, and Lac⁺ 5FUR^R unselected chromosomal mutants display similar frequencies of transfer for all selections tested. This indicates that the state of the F' is the same in all three of these different populations. Therefore, the generation of an adaptive Lac⁺ revertant and/or an unselected mutations does not result in a stable change in state of the F' (i.e. F' to Hfr or vice versa). The isolates of all three populations tested displayed a much greater frequency of transfer of proline

prototrophy than that observed for the Hfr control strain whose origin of transfer is close to the *proAB* locus (Table A-1). In addition, the frequency of transfer of proline prototrophy was the greatest amongst all the selections tested for all three tested populations (Table A-1). This indicates that all three of these tested populations contain a significant number of F' cells, as the F' of FC40 carries the *proAB-lac* region that has been deleted from the chromosome (Cairns and Foster, 1991), such that one would expect a high frequency of transfer of proline prototrophy compared with chromosomal markers. The frequency of transfer of proline prototrophy for all three tested populations is also unaffected after 200 minutes of mating to a *recA* recipient (Table A-2), which further supports the existence of F' cells. However, some of the data of Table A-1 and Table A-2 indicate that the FC40, Lac⁺ revertant, and 5FUR^R isolates are Hfr strains or a proportion of the population of cells exists as Hfr's. The frequency of transfer of arginine for all three populations is as high or higher than that for the Hfr control strain in which the origin of transfer is close to *argE* (Table A-1), which resides at 89.4 minutes on the *E. coli* chromosome. *upp* resides at 56.2 minutes on the *E. coli* chromosome, so there is no obvious correlation between the high rate of transfer of *argE* and the position of *upp* on the chromosome. Furthermore, the frequency of transfer to a *recA* recipient compared to a *rec*⁺ recipient is considerably decreased in the three populations for all of the selections except *pro*⁺ (Table A-2), indicating that a number of cells in all three populations are Hfr's. Whether they have become stable Hfr's or whether the population is transiently shifting between Hfr and F' is unknown. Transitory Hfr cells have been shown to exist in a population derived from an F' parent (Pittard and Adelberg, 1963a,b). The high frequency of transfer of proline prototrophy

suggest, however, that the majority of cells in the populations tested are F' cells.

These results do not reveal any correlation of either chromosomal mutation or F' *lac* reversion with stable Hfr formation. The data can not rule out the possibility that transient Hfr's form. It is also possible that a subpopulation of cells are always in the state of being Hfr or are transiently shifting between Hfr and F'. This could be the subpopulation that gives rise to adaptive mutations in our system. To determine whether this is the case, the hypermutable subpopulation should be isolated and it should be determined whether or not the cells in this subpopulation exist as F' cells or as Hfr's.

Materials and Methods

Equal volumes of mid-log phase donor and saturated recipient, AB1157, were mixed in fresh prewarmed LBH 0.2% glucose in which both the donor and recipient cells can grow. This mating mix was incubated without shaking at 37°C for 20 and 200 minutes. After 20 or 200 minutes the mating mix was washed twice in minimal M9 salts, at which point dilution's were made and 10 μ l of these were spotted onto media selective for transconjugants. Selective media consisted of M9 thiamine 100 μ g/ml streptomycin 50 μ g/ml histidine, leucine, threonine, proline, and arginine. Media selective for proline, histidine or arginine auxotrophy lacked these amino acids, respectively, and contained 0.2% glucose as the carbon source. Media selective for the ability to ferment xylose and galactose contained 0.2% of those sugars, respectively, as the carbon source. The plates were incubated overnight at 37°C and colonies

scored. Viable counts were made of the donor and the frequency of transfer was calculated as number of transconjugants per donor cell.

Table A-1. FC40, Lac⁺ revertants and unselected mutants do not appear to be stable Hfr strains.

Selection & time allowed for mating	FC40 (n ^a =6)	Lac ⁺ revertants (n=10)	Unselected 5FU ^R mutants (n=10)	Hfr strain:			F ⁺ d strain (n=4)
				Hfr strain ^c : oriT close to selected locus (n=4)	Hfr strain: oriT far from selected locus (n=4)	No. of trans-conjugants per donor cell	
	No. of trans-conjugants per donor cell [Mean(±SE)] ^b	No. of trans-conjugants per donor cell [Mean(±SE)]	No. of trans-conjugants per donor cell [Mean(±SE)]	No. of trans-conjugants per donor cell [Mean(±SE)]	No. of trans-conjugants per donor cell [Mean(±SE)]	No. of trans-conjugants per donor cell [Mean(±SE)]	No. of trans-conjugants per donor cell [Mean(±SE)]
Sm ^R Pro ⁺ 20 mins	1.6x10 ⁻³ (4.4x10 ⁻⁴)	1.7x10 ⁻³ (1.9x10 ⁻⁵)	1.6x10 ⁻³ (2.2x10 ⁻⁵)	1.1x10 ⁻⁸ (0.0)	5.2x10 ⁻⁸ (8.9x10 ⁻⁹)	2.3x10 ⁻⁷ (5.0x10 ⁻⁸)	
Sm ^R Pro ⁺ 200 mins	5.2x10 ⁻² (3.6x10 ⁻³)	1.9x10 ⁻² (2.7x10 ⁻²)	2.2x10 ⁻² (2.0x10 ⁻²)	3.0x10 ⁻⁸ (0.0)	8.5x10 ⁻⁷ (1.5x10 ⁻⁸)	3.7x10 ⁻⁶ (3.7x10 ⁻⁶)	
Sm ^R Arg ⁺ 20 mins	3.3x10 ⁻⁶ (4.0x10 ⁻⁷)	4.5x10 ⁻⁶ (0.0)	4.8x10 ⁻⁶ (0.0)	6.5x10 ⁻⁷ (0.0)	1.4x10 ⁻⁸ (0.0)	1.3x10 ⁻⁸ (0.0)	
Sm ^R Arg ⁺ 200 mins	6.2x10 ⁻⁵ (2.7x10 ⁻⁶)	7.7x10 ⁻⁵ (0.0)	6.1x10 ⁻⁵ (0.0)	1.6x10 ⁻⁵ (6.0x10 ⁻⁶)	8.5x10 ⁻⁸ (0.0)	1.3x10 ⁻⁷ (1.1x10 ⁻⁸)	

Table A-1 continued...

Selection & time allowed for mating	FC40 (n=6)	Lac ⁺ revertants (n=10)	Unselected 5FU ^R mutants (n=10)	Hfr strain: <i>oriT</i> close to locus (n=4)	Hfr strain: <i>oriT</i> far from locus (n=4)	F ⁺ strain (n=4)
Sm ^R Xyl ⁺ 20 mins	6.2x10 ⁻⁷ (7.8x10 ⁻⁸)	5.0x10 ⁻⁷ (0.0)	3.0x10 ⁻⁷ (0.0)	1.7x10 ⁻⁴ (2.5x10 ⁻⁶)	0 (0.0)	8.5x10 ⁻⁹ (0.0)
Sm ^R Xyl ⁺ 200 mins	3.7x10 ⁻⁵ (3.7x10 ⁻⁶)	2.4x10 ⁻⁵ (2.0x10 ⁻⁷)	1.8x10 ⁻⁵ (1.3x10 ⁻⁷)	3.2x10 ⁻⁴ (3.0x10 ⁻⁵)	6.2x10 ⁻⁷ (0.0)	4.6x10 ⁻⁷ (0.0)
Sm ^R His ⁺ 20 mins	5.5x10 ⁻⁷ (7.5x10 ⁻⁸)	4.3x10 ⁻⁷ (0.0)	1.3x10 ⁻⁷ (0.0)	2.0x10 ⁻⁵ (0.0)	2.6x10 ⁻⁵ (2.1x10 ⁻⁵)	7.8x10 ⁻⁸ (0.0)
Sm ^R His ⁺ 200 mins	5.2x10 ⁻⁶ (1.5x10 ⁻⁷)	4.1x10 ⁻⁶ (0.0)	2.8x10 ⁻⁶ (0.0)	1.9x10 ⁻⁴ (6.1x10 ⁻⁷)	2.3x10 ⁻⁴ (9.6x10 ⁻⁶)	2.4x10 ⁻⁶ (2.7x10 ⁻⁷)
Sm ^R Gal ⁺ 20 mins	1.8x10 ⁻⁷ (3.6x10 ⁻⁸)	5.4x10 ⁻⁷ (0.0)	3.8x10 ⁻⁷ (0.0)	3.2x10 ⁻⁸ (0.0)	1.3x10 ⁻⁶ (0.0)	8.5x10 ⁻⁸ (0.0)
Sm ^R Gal ⁺ 200 mins	5.0x10 ⁻⁶ (2.7x10 ⁻⁷)	3.3x10 ⁻⁶ (9.7x10 ⁻⁹)	2.3x10 ⁻⁶ (8.6x10 ⁻⁹)	8.3x10 ⁻⁷ (0.0)	2.8x10 ⁻⁵ (0.0)	2.6x10 ⁻⁶ (1.1x10 ⁻⁸)

Donor strains are FC40, Lac⁺ revertants, Lac⁺ 5FUR mutants, and control Hfr and F⁺ strains. The recipient is AB1157 *thr1 ara1-4 leuB6 lacY1 glnV44 galK2 hisG4 rpsL31 xylA5 mtl1 argE3 thi-1 Δ(gpt-proA) tsx-33 gsrO LAM⁻ rac-O rfbD1 mgl-51 kdgK51*

^a n, number of independent isolates tested.

^b SE, one standard error of the mean.

^c Hfr control strains for each selection consisted of one in which origin of transfer was close to the locus under selection (e.g. *proA*) such that transfer of that locus by the Hfr strain to the recipient AB1157 would occur early in the mating process. The second Hfr strain was such that the origin of transfer was farther from the locus under selection, such that transfer of that locus by the Hfr strain to the recipient AB1157 would occur late in the mating process.

Hfr strains: BW6166 (SMR3389) (Arg⁺ & Pro⁺), BW6175 (SMR3394) (Xyl⁺ & His⁺), NK6051 (SMR3396) (Pro⁺ & Arg⁺), BW6160 (SMR3398) (Gal⁺ & Xyl⁺), and BW5660 (SMR3400) (His⁺ & Gal⁺).

^d F⁺ control strain, R881 (SMR3290).

Table A-2. Mating into a *recA* recipient.

Selection & time allowed for mating	Hfr strain:					
	FC40 (n=6)	Lac ⁺ revertants (n=10)	Unselected 5FU ^R mutants (n=10)	Hfr strain: <i>oriT</i> close to selected locus (n=4)	<i>oriT</i> far from selected locus (n=4)	F ⁺ strain (n=4)
	No. of trans-conjugants per donor cell [Mean(±SE)]	No. of trans-conjugants per donor cell [Mean(±SE)]	No. of trans-conjugants per donor cell [Mean(±SE)]	No. of trans-conjugants per donor cell [Mean(±SE)]	No. of trans-conjugants per donor cell [Mean(±SE)]	No. of trans-conjugants per donor cell [Mean(±SE)]
Sm ^R Pro ⁺ 20 mins	3.3x10 ⁻⁵ (4.1x10 ⁻⁶)	1.9x10 ⁻⁵ (8.1x10 ⁻⁶)	1.2x10 ⁻⁵ (3.3x10 ⁻⁶)	0.0 (0.0)	3.2x10 ⁻⁸ (3.2x10 ⁻⁸)	1.0x10 ⁻⁷ (1.0x10 ⁻⁷)
Sm ^R Pro ⁺ 200 mins	5.3x10 ⁻² (4.2x10 ⁻³)	2.7x10 ⁻² (1.0x10 ⁻²)	2.0x10 ⁻² (1.3x10 ⁻³)	0.0 (0.0)	0.0 (0.0)	4.1x10 ⁻⁷ (4.1x10 ⁻⁷)
Sm ^R Arg ⁺ 20 mins	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Sm ^R Arg ⁺ 200 mins	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

Table A-2 continued...

Selection & time allowed for mating	FC40 (n=6)	Lac ⁺ revertants (n=10)	Unselected 5FU ^R mutants (n=10)	Hfr strain: <i>oriT</i> close to locus (n=4)	Hfr strain: <i>oriT</i> far from locus (n=4)	F ⁺ strain (n=4)
Sm ^R Xyl ⁺ 20 mins	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0 (0.0)	0.0 (0.0)
Sm ^R Xyl ⁺ 200 mins	2.3x10 ⁻⁷ (2.4x10 ⁻⁶)	2.0x10 ⁻⁷ (3.6x10 ⁻⁶)	1.3x10 ⁻⁷ (2.4x10 ⁻⁶)	2.0x10 ⁻⁶ (9.8x10 ⁻⁶)	6.2x10 ⁻⁷ (0.0)	0.0 (0.0)
Sm ^R His ⁺ 20 mins	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	6.2x10 ⁻⁷ (1.8x10 ⁻⁷)	0.0 (0.0)
Sm ^R His ⁺ 200 mins	8.7x10 ⁻⁸ (8.7x10 ⁻⁸)	0.0 (0.0)	0.0 (0.0)	6.1x10 ⁻⁷ (1.1x10 ⁻⁷)	1.2x10 ⁻⁷ (1.2x10 ⁻⁷)	0.0 (0.0)
Sm ^R Gal ⁺ 20 mins	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Sm ^R Gal ⁺ 200 mins	1.2x10 ⁻⁸ (7.7x10 ⁻⁹)	4.5x10 ⁻⁹ (0.0)	8.6x10 ⁻⁹ (0.0)	0.0 (0.0)	2.9x10 ⁻⁷ (7.6x10 ⁻⁸)	0.0 (0.0)

Conditions of mating and strains are as per Table A-1 except recipient strain is AB1157*recA*.

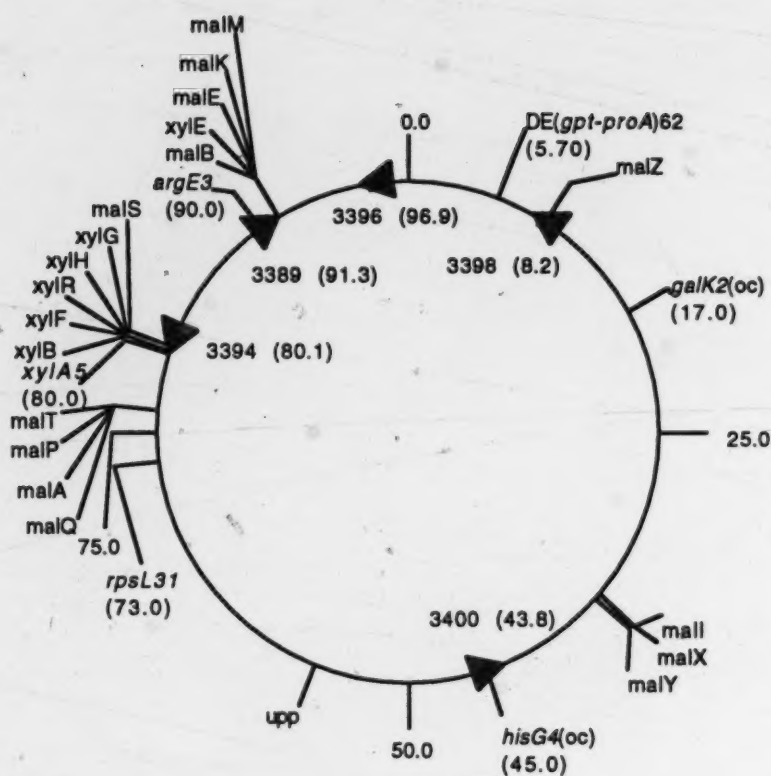


Figure A-1. Site map of *E. coli* chromosome. Mutant loci of AB1157 used in mating experiments are italicized and their positions on the chromosome are mapped in minutes. Hfr strain numbers are marked and insertion points with locations mapped in minutes are indicated by the arrows.

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Appendix B
Unselected mutant collection

Table B-1. UNSELECTED MUTANT COLLECTION

Collection ¹ #	Mutant Phenotype ²	Expt#/culture/ day isolated ³	original (O), replica (R) or purified (P) mutant colony	Notes
1	5FC ^R	15/11/a	O	pure colony
2	5FC ^R	15/11/a	P	mapped to <i>codAB</i>
3	Lac ⁻ 5FC ^R	16/6/na	O	stressed, sectored
4	Lac ⁻ 5FC ^R	16/6/na	P	stressed
5	5FC ^R	16/16/a	O	pure colony
6	5FC ^R	16/16/a	P	mapped to <i>codAB</i>
7	5FC ^R	16/14/a	O	sectored colony
8	5FC ^R	16/14/a	P	
9	Tet ^R	17/1/a	R	
10	Tet ^R	17/4/a	R	
11	Tet ^R	17/4/nd	R	
12	Tet ^R	17/6/a	R	mapped to pW17; sequenced -1G in run of 6G's
13	Tet ^R	17/6/a	R	
14	Tet ^R	17/6/a	R	
15	Tet ^R	17/7/a	R	
16†	Tet ^R	17/8/gd	R	
17	Tet ^R	17/8/a	R	mapped to pW17
18	Tet ^R	17/8/a	R	mapped to pW17
19	Tet ^R	17/9/a	R	
20	Tet ^R	17/9/a	R	mapped to pW17; sequenced -1G in run of 6G's

¹ † denotes those mutants that were not reported in chapter 2.

² Strain background is FC40 except for Tet^R isolates which are strain FC40[pW17](SMR#1401), unless otherwise noted. Strains are all Lac⁺ except where noted as Lac⁻. 5-fluorocytosine resistant, 5FC^R; Tetracycline resistant, Tet^R; 5-flourouracil resistant, 5FUR^R; temperature sensitive, Ts; maltose, xylose, fructose fermentation defective, Mal⁻, Xyl⁻, and Fruc⁻ respectively; amino acid auxotroph, aa aux. Isolates #278 to 300 are in background DH5 α and were used for sequencing; Tet^R pW17 plasmid DNA was isolated from FC40[pW17] strains and transformed into DH5 α .

³ Recorded either as a number or one of the following: a, adaptive; gd, growth-dependent; na, not applicable; nd, not determined; JN, Jayan Nagendran.

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
21	Tet ^R	17/9/nd	R	sequenced -1G in run of 6G's
22	Tet ^R	17/10/a	R	
23	Tet ^R	17/10/a	R	
24	Tet ^R	17/10/a	R	sequenced -1G in run of 6G's
25	Tet ^R	17/11/a	R	
26	Tet ^R	17/11/a	R	
27	Tet ^R	17/12/a	R	sequenced -1G in run of 6G's
28	Tet ^R	17/13/a	R	
29	Tet ^R	17/13/a	R	
30	Tet ^R	17/13/a	R	sequenced -1G in run of 6G's
31	Tet ^R	17/14/a	R	
32	Tet ^R	17/14/a	R	
33	Tet ^R	17/14/a	R	
34	Tet ^R	17/16/a	R	
35	Tet ^R	17/17/nd	R	
36	Tet ^R	17/17/a	R	
37	Tet ^R	17/17/nd	R	
38	Tet ^R	17/18/a	R	
39	Tet ^R	17/18/a	R	
40	Tet ^R	17/19/a	R	
41	Tet ^R	17/19/a	R	
42	Tet ^R	17/20/a	R	
43	Tet ^R	17/20/a	R	
44	Tet ^R	17/21/a	R	
45	Tet ^R	17/22/a	R	sequenced -1G in run of 6G's
46	Tet ^R	17/22/a	R	
47	Tet ^R	17/24/a	R	
48	Tet ^R	17/24/a	R	
49	Tet ^R	17/24/a	R	
50†	Tet ^R	17/24/gd	R	
51	Tet ^R	17/24/a	R	
52	Tet ^R	17/24/a	R	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
53†	Tet ^R	17/25/gd	R	
54	Tet ^R	17/25/a	R	
55	Tet ^R	17/26/a	R	
56	Tet ^R	17/27/a	R	
57	Tet ^R	17/28/a	R	
58†	Tet ^R	17/28/gd	R	
59†	Tet ^R	17/28/gd	R	
60†	Tet ^R	17/28/gd	R	
61	Tet ^R	17/28/a	R	
62	Tet ^R	17/28/nd	R	
63	Tet ^R	17/6/a	P	
64	Tet ^R	17/6/a	P	
65	Tet ^R	17/6/a	P	
66	Tet ^R	17/8/a	P	
67	Tet ^R	17/8/a	P	
68	Tet ^R	17/9/a	P	
69	Tet ^R	17/9/a	P	
70†	Tet ^R	17/9/gd	P	
71	Tet ^R	17/11/a	P	
72	Tet ^R	17/12/a	P	
73	Tet ^R	17/13/a	P	
74	Tet ^R	17/13/a	P	
75	Tet ^R	17/14/a	P	
76	Tet ^R	17/16/a	P	
77	Tet ^R	17/22/a	P	mutator mapped to <i>mutL</i>
78	Tet ^R	17/22/a	P	
79	Tet ^R	17/22/a	P	
80	Tet ^R	17/22/a	P	
81	Tet ^R	17/22/a	P	
82	Tet ^R	17/22/a	P	
83	Tet ^R	17/22/a	P	
84	Tet ^R	17/25/a	P	mutator mapped to <i>mutL</i>
85	Tet ^R	17/25/a	P	
86	Tet ^R	17/25/a	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
87	Tet ^R	17/28/a	P	
88	Tet ^R	17/6/a	O	
89	Tet ^R	17/6/a	O	
90	Tet ^R	17/6/a	O	
91	Tet ^R	17/8/a	O	
92	Tet ^R	17/8/a	O	
93	Tet ^R	17/9/a	O	
94	Tet ^R	17/9/a	O	
95†	Tet ^R	17/9/gd	O	
96	Tet ^R	17/11/a	O	
97	Tet ^R	17/12/a	O	
98	Tet ^R	17/13/a	O	
99	Tet ^R	17/13/a	O	
100	Tet ^R	17/14/a	O	
101	Tet ^R	17/16/a	O	
102	Tet ^R	17/22/a	O	
103	Tet ^R	17/22/a	O	
104	Tet ^R	17/22/a	O	
105	Tet ^R	17/22/a	O	
106	Tet ^R	17/22/a	O	
107	Tet ^R	17/22/a	O	
108	Tet ^R	17/22/a	O	
109	Tet ^R	17/25/a	O	
110	Tet ^R	17/25/a	O	
111	Tet ^R	17/25/a	O	
112	Tet ^R	17/28/a	O	
113†	5FC ^R	18/4/gd	O	pure colony; mapped to <i>codAB</i>
114†	5FC ^R	18/4/gd	O	pure colony
115	5FC ^R	18/4/a	O	pure colony; mapped to <i>codAB</i>
116	5FC ^R	18/4/a	O	pure colony; mapped to <i>codAB</i>
117†	5FC ^R	18/4/gd	O	pure colony; mapped to <i>codAB</i>
118	5FU ^R	18/13/a	O	pure colony
119	5FC ^R	18/16/a	O	pure colony

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
120†	Ts (Lac)	18/3/6	O	Ts on Lac only
121†	Ts (Lac)	18/5/6	O	Ts on Lac only
122†	Ts (Lac)	18/6/5	O	Ts on Lac only
123†	Ts (Lac)	18/8/5	O	Ts on Lac only
124†	Ts (Lac)	18/8/5	O	Ts on Lac only
125†	Ts (Lac)	18/9/6	O	Ts on Lac only
126†	Ts (Lac)	18/9/6	O	Ts on Lac only
127†	Ts (Lac)	18/9/4	O	Ts on Lac only
128†	Ts (Lac)	18/9/5	O	Ts on Lac only
129†	Ts (Lac)	18/10/4	O	Ts on Lac only
130†	Ts (Lac)	18/11/6	O	Ts on Lac only
131†	Ts (Lac)	18/12/6	O	Ts on Lac only
132†	Ts (Lac)	18/12/5	O	Ts on Lac only
133†	Ts (Lac)	18/12/3	O	Ts on Lac only
134†	Ts (Lac)	18/13/3	O	Ts on Lac only
135†	Ts (Lac)	18/14/5	O	Ts on Lac only
136†	Ts (Lac)	18/14/6	O	Ts on Lac only
137†	Ts (Lac)	18/14/4	O	Ts on Lac only
138†	Ts (Lac)	18/14/5	O	Ts on Lac only
139†	Ts (Lac)	18/14/6	O	Ts on Lac only
140†	Ts (Lac)	18/14/3	O	Ts on Lac only
141†	Ts (Lac)	18/14/5	O	Ts on Lac only
142†	Ts (Lac)	18/14/5	O	Ts on Lac only
143†	Ts (Lac)	18/14/6	O	Ts on Lac only
144†	Ts (Lac)	18/14/6	O	Ts on Lac only
145†	Ts (Lac)	18/15/3	O	Ts on Lac only
146†	Ts (Lac)	18/15/6	O	Ts on Lac only
147†	Ts (Lac)	18/15/2	O	Ts on Lac only
148†	Ts (Lac)	18/15/5	O	Ts on Lac only
149†	Ts (Lac)	18/15/6	O	Ts on Lac only
150†	Ts (Lac)	18/16/3	O	Ts on Lac only
151†	Ts (Lac)	18/17/3	O	Ts on Lac only
152†	Ts (Lac)	18/17/5	O	Ts on Lac only
153†	Ts (Lac)	18/17/6	O	Ts on Lac only
154†	Ts (Lac)	18/18/6	O	Ts on Lac only
155†	Ts (Lac)	18/18/5 or 6	O	Ts on Lac only
156†	Ts (Lac)	18/18/4	O	Ts on Lac only
157†	Ts (Lac)	18/18/6	O	Ts on Lac only
158†	Ts (Lac)	18/18/2	O	Ts on Lac only
159†	Ts (Lac)	18/19/3	O	Ts on Lac only
160†	Ts (Lac)	18/20/3	O	Ts on Lac only

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
161†	Xyl ⁻	18/4/2	O	
162†	Xyl ⁻	18/4/2	O	
163	Xyl ⁻	18/4/6	O	
164	Lac ⁻ 5FCR	19/15/5	O	stressed
165†	Lac ⁻ aa aux	19/23/5	O	stressed
166	Lac ⁻ Tet ^R	20/14/na	P	unstressed
167	Lac ⁻ Tet ^R	20/14/na	P	unstressed; sequenced -1G in run of 6G's
168	Lac ⁻ Tet ^R	20/15/na	P	unstressed
169†	aa aux	19/1/5	O	
170†	aa aux	19/4/4	O	
171†	aa aux	19/13/5	O	
172†	aa aux	19/1/5	P	
173†	aa aux	19/4/4	P	
174†	aa aux	19/13/5	P	
175	Tet ^R	20/2/6	R	sequenced -1G in run of 6G's
176	Tet ^R	20/6/4	R	sequenced -1G in run of 6G's
177	Tet ^R	20/10/4	R	mapped to pW17; sequenced -1G in run of 6G's
178	Tet ^R	20/10/5	R	mapped to pW17; sequenced -1G in run of 6G's
179†	Tet ^R	20/11/2	R	mapped to pW17; sequenced -1G in run of 6G's
180	Tet ^R	20/10/4	R	mapped to pW17; sequenced -1G in run of 6G's
181	Tet ^R	20/10/5	R	mapped to pW17; sequenced -1G in run of 6G's
182	Tet ^R	20/10/6	R	mapped to pW17; sequenced -1G in run of 6G's
183	Tet ^R	20/10/5	R	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
184	Tet ^R	20/10/3	R	sequenced -1G in run of 6G's
185†	Ts (Lac)	19/1/4	P	Ts on Lac only
186†	Ts (Lac)	19/1/4	P	Ts on Lac only
187†	Ts (Lac)	19/1/5	P	Ts on Lac only
188†	Ts (Lac)	19/3/4	P	Ts on Lac only
189†	Ts (Lac)	19/4/4	P	Ts on Lac only
190†	Ts (Lac)	19/4/5	P	Ts on Lac only
191†	Ts (Lac)	19/5/5	P	Ts on Lac only
192†	Ts (Lac)	19/5/5	P	Ts on Lac only
193†	Ts (Lac)	19/6/4	P	Ts on Lac only
194†	Ts (Lac)	19/8/5	P	Ts on Lac only
195†	Ts (Lac)	19/9/5	P	Ts on Lac only
196†	Ts (Lac)	19/9/5	P	Ts on Lac only
197†	Ts (Lac)	19/9/4	P	Ts on Lac only
198†	Ts (Lac)	19/9/5	P	Ts on Lac only
199	5FCR	19/1/4	O	sectoried colony
200	5FCR	19/1/5	O	pure colony
201	5FCR	19/1/5	O	sectoried colony
202	5FCR	19/2/4	O	pure colony; mapped to <i>codAB</i>
203	5FCR	19/3/5	O	pure colony; mapped to <i>codAB</i>
204	5FCR	19/4/3	O	pure colony
205	5FCR	19/5/3	O	pure colony
206	5FCR	19/6/3	O	pure colony
207	5FCR	19/6/5	O	pure colony; mapped to <i>codAB</i>
208	5FCR	19/6/4	⊙	pure colony
209	5FCR	19/6/4 or 5	O	pure colony
210†	5FCR	19/8/2	O	sectoried colony
211	5FCR	19/8/5	O	pure colony; mapped to <i>codAB</i>
212	5FCR	19/10/4	O	sectoried colony
213	5FCR	19/10/nd	O	sectoried colony
214	5FCR	19/12/5	O	pure colony
215†	5FCR	19/13/2	O	pure colony
216†	5FCR	19/13/2	O	pure colony
217†	5FCR	19/13/2	O	pure colony

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
218†	5FCR	19/13/2	O	pure colony
219†	5FCR	19/13/2	O	pure colony
220	5FUR	19/14/5	O	pure colony; mapped to <i>upp</i>
221	5FCR	19/15/4	O	pure colony
222	5FCR	19/15/5	O	sectoried colony
223	5FCR	19/17/4	O	pure colony
224	5FCR	19/17/5	O	pure colony
225	5FCR	19/19/5	O	pure colony
226	5FCR	19/19/5	O	pure colony
227	5FCR	19/20/3	O	pure colony
228	5FCR	19/21/5	O	pure colony
229	5FCR	19/21/nd	O	sectoried colony
230	5FCR	19/22/5	O	pure colony; mapped to <i>codAB</i>
231	5FCR	19/22/4	O	pure colony; mapped to <i>codAB</i>
232	5FCR	19/22/5	O	pure colony
233	5FCR	19/22/5	O	sectoried colony
234	5FCR	19/22/4 or 5	O	sectoried colony
235	5FCR	19/23/4	O	pure colony; mapped to <i>codAB</i>
236	5FCR	19/23/4	O	pure colony
237†	5FCR	18/4/gd	P	
238†	5FCR	18/4/gd	P	
239	5FCR	18/4/a	P	
240	5FCR	18/4/a	P	
241†	5FCR	18/4/gd	P	
242	5FUR	18/13/a	P	
243	5FCR	18/16/a	P	
244	5FCR	19/1/4	P	
245	5FCR	19/1/5	P	
246	5FCR	19/1/5	P	
247	5FCR	19/2/4	P	
248	5FCR	19/3/5	P	
249	5FCR	19/4/3	P	
250	5FCR	19/5/3	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
251	5FCR	19/6/3	P	
252	5FCR	19/6/5	P	
253	5FCR	19/6/4	P	
254	5FCR	19/6/4 or 5	P	
255†	5FCR	19/8/2	P	
256	5FCR	19/8/5	P	
257	5FCR	19/10/4	P	
258	5FCR	19/10/nd	P	
259	5FCR	19/12/5	P	
260†	5FCR	19/13/2	P	
261	5FUR	19/14/5	P	
262	5FCR	19/15/4	P	
263	5FCR	19/15/5	P	
264	5FCR	19/17/4	P	
265	5FCR	19/17/5	P	
266	5FCR	19/19/5	P	
267	5FCR	19/19/5	P	
268	5FCR	19/20/3	P	
269	5FCR	19/21/5	P	
270	5FCR	19/21/nd	P	
271	5FCR	19/22/5	P	
272	5FCR	19/22/4	P	
273	5FCR	19/22/5	P	
274	5FCR	19/22/5	P	
275	5FCR	19/22/4 or 5	P	
276	5FCR	19/23/4	P	
277	5FCR	19/23/4	P	
278	Tet ^R	na	na	pW17 from #12
279	Tet ^R	na	na	pW17 from #18
280	Tet ^R	na	na	pW17 from #20
281	Tet ^R	na	na	pW17 from #25
282	Tet ^R	na	na	pW17 from #26
283	Tet ^R	na	na	pW17 from #27
284	Tet ^R	na	na	pW17 from #29
285	Tet ^R	na	na	pW17 from #30
286	Tet ^R	na	na	pW17 from #34

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
287	Tet ^R	na	na	pW17 from #45
288	Tet ^R	na	na	pW17 from #175
289	Tet ^R	na	na	pW17 from #176
290	Tet ^R	na	na	pW17 from #177
291	Tet ^R	na	na	pW17 from #178
292	Tet ^R	na	na	pW17 from #179
293	Tet ^R	na	na	pW17 from #180
294	Tet ^R	na	na	pW17 from #181
295	Tet ^R	na	na	pW17 from #182
296	Tet ^R	na	na	pW17 from #183
297	Tet ^R	na	na	pW17 from #184
298	Tet ^R	na	na	pW17 from #166
299	Tet ^R	na	na	pW17 from #167
300	Tet ^R	na	na	pW17 from #168
301†	5FCR	21/1/2	P	mapped to <i>codAB</i>
302	5FCR	21/1/3	P	mapped to <i>codAB</i>
303	5FCR	21/1/5	P	mapped to <i>codAB</i>
304	5FCR	21/2/4	P	mapped to <i>codAB</i>
305	5FCR	21/2/4	P	mapped to <i>codAB</i>
306	5FCR	21/2/5	P	mapped to <i>codAB</i>
307	5FCR	21/2/5	P	mapped to <i>codAB</i>
308	5FCR	21/2/5	P	mapped to <i>codAB</i>
309	5FCR	21/2/4	P	mapped to <i>codAB</i>
310	5FCR	21/2/3	P	mapped to <i>codAB</i>
311†	5FCR	21/2/2	P	mapped to <i>codAB</i>
312	5FCR	21/3/5	P	mapped to <i>codAB</i>
313	5FUR	21/3/5	P	mapped to <i>upp</i>
314	5FUR	21/3/5	P	mapped to <i>upp</i>
315	5FUR	21/3/5	P	mapped to <i>upp</i>
316	5FCR	21/3/4	P	
317	5FCR	21/4/3	P	
318	5FCR	21/4/4	P	
319	5FCR	21/4/4	P	
320	5FCR	21/4/4	P	
321	5FCR	21/4/4	P	
322	5FCR	21/4/3	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
323	5FCR	21/4/4	P	
324	5FCR	21/4/5	P	
325	5FCR	21/4/5	P	
326	5FCR	21/4/5	P	
327	5FCR	21/4/4	P	
328†	5FCR	21/5/2	P	
329	5FUR	21/5/3	P	mapped to <i>upp</i>
330	5FUR	21/6/4	P	mapped to <i>upp</i>
331	5FCR	21/6/4	P	
332	5FCR	21/6/5	P	
333	5FUR	21/7/4	P	mapped to <i>upp</i>
334	5FCR	21/7/4	P	
335	5FUR	21/7/4	P	mapped to <i>upp</i>
336	5FCR	21/7/5	P	
337	5FCR	21/7/4	P	
338	5FUR	21/7/4	P	mapped to <i>upp</i>
339	5FCR	21/9/3	P	
340	5FCR	21/10/3	P	
341	5FCR	21/10/3	P	
342	5FCR	21/10/4	P	
343	5FCR	21/10/3	P	
344	5FUR	21/11/3	P	mapped to <i>upp</i>
345	5FCR	21/11/3	P	
346	5FCR	21/11/3	P	
347	5FUR	21/11/4	P	
348†	5FCR	21/11/2	P	mapped to <i>upp</i>
349	5FCR	21/11/5	P	
350	5FUR	21/11/5	P	mapped to <i>upp</i>
351	5FCR	21/11/4	P	
352	5FCR	21/12/4	P	
353	5FCR	21/12/4	P	
354	5FCR	21/12/5	P	
355	5FCR	21/12/4	P	
356	5FCR	21/12/3	P	
357	5FCR	21/12/3	P	
358	5FCR	21/12/3	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
359	5FCR	21/13/4	P	
360	5FCR	21/14/3	P	
361	5FCR	21/14/3	P	
362	5FCR	21/15/5	P	
363	5FUR	21/15/4	P	mapped to <i>upp</i>
364	5FCR	21/16/5	P	
365	5FCR	21/16/3	P	
366	5FCR	21/17/5	P	
367	5FCR	21/17/5	P	
368	5FCR	21/17/4	P	
369	5FCR	21/17/3	P	
370	5FCR	21/17/3	P	
371	5FCR	21/17/3	P	
372				excluded
373	5FCR	21/17/4	P	
374	5FCR	21/17/3	P	
375				excluded
376	5FCR	21/17/3	P	
377	5FCR	21/17/3	P	
378	5FCR	21/18/4	P	
379	5FCR	21/18/4	P	
380	5FCR	21/18/4	P	
381	5FCR	21/18/5	P	
382†	5FCR	21/18/2	P	
383	5FCR	21/18/5	P	
384	5FCR	21/18/5	P	
385	5FCR	21/18/4	P	
386	5FUR	21/18/3	P	mapped to <i>upp</i>
387	5FCR	21/18/4	P	
388	5FCR	21/18/4	P	
389	5FCR	21/18/5	P	
390	5FCR	21/19/4	P	
391	5FCR	21/20/4	P	
392	5FCR	21/20/3	P	mapped to <i>codAB</i>
393	5FCR	21/20/5	P	
394	5FCR	21/20/3	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
395	5FCR	21/20/5	P	
396	5FUR	21/20/4	P	mapped to <i>upp</i>
397	5FUR	21/20/5	P	mapped to <i>upp</i>
398	Lac ⁻ 5FCR	21/20/na	P	unstressed
399	Lac ⁻ 5FCR	22/81/4	P	stressed; mapped to <i>codAB</i>
400	Lac ⁻ 5FCR	22/67/na	P	unstressed; mapped to <i>codAB</i>
401	Lac ⁻ Fruc ⁻	22/51/5	P	stressed
402	Lac ⁻ Xyl ⁻	22/23/5	P	stressed
403	Lac ⁻ Xyl ⁻	22/50/5	P	stressed
404	Lac ⁻ Xyl ⁻	22/80/5	P	stressed
405	Lac ⁻ Xyl ⁻	22/53/6	P	stressed; #405 to #412 are from the same plate and likely clones
406	Lac ⁻ Xyl ⁻	22/53/6	P	stressed
407	Lac ⁻ Xyl ⁻	22/53/6	P	stressed
408	Lac ⁻ Xyl ⁻	22/53/6	P	stressed
409	Lac ⁻ Xyl ⁻	22/53/6	P	stressed
410	Lac ⁻ Xyl ⁻	22/53/6	P	stressed
411	Lac ⁻ Xyl ⁻	22/53/6	P	stressed
412	Lac ⁻ Xyl ⁻	22/53/6	P	stressed
413	Lac ⁻ Fruc ⁻	22/64/na	P	unstressed
414	Lac ⁻ Ts	21/7/na	P	unstressed
415	Lac ⁻ Ts	21/12/5	P	stressed
416	Lac ⁻ Ts	21/7/na	O	unstressed
417	Lac ⁻ Ts	21/12/5	O	stressed
418				excluded
419				excluded
420	Xyl ⁻	22/51/nd	P	mapped to chromosome
421	Xyl ⁻	22/51/nd	P	mapped to chromosome
422	Mal ⁻	22/52/nd	P	
423	Mal ⁻	22/53/nd	P	mapped to chromosome

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
424	Mal ⁻	22/53/nd	P	mutator mapped to <i>mutL</i>
425				excluded
426				excluded
427	Mal ⁻	22/56/nd	P	
428				excluded
429	Mal ⁻	22/57/nd	P	
430	Mal ⁻	22/57/nd	P	
431	Xyl ⁻	22/58/nd	P	
432	Mal ⁻	22/59/nd	P	
433	Xyl ⁻	22/61/nd	P	mapped to chromosome
434				excluded
435	Xyl ⁻	22/62/nd	P	mapped to chromosome
436	Mal ⁻	22/63/nd	P	mapped to chromosome
437				excluded
438				excluded
439				excluded
440				excluded
441				excluded
442	Xyl ⁻	22/70/nd	P	mapped to chromosome
443	Mal ⁻	22/70/nd	P	mapped to chromosome
444	Mal ⁻	22/71/nd	P	mapped to chromosome
445	Mal ⁻	22/71/nd	P	
446	Xyl ⁻	22/73/nd	P	mapped to chromosome
447	Mal ⁻	22/73/nd	P	
448				excluded
449				excluded
450				excluded
451				excluded
452	Mal ⁻	22/79/nd	P	mapped to chromosome
453	Xyl ⁻	22/80/nd	P	mapped to chromosome

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
454	Xyl ⁻	22/80/nd	P	mapped to chromosome
455	Mal ⁻	22/81/nd	P	mapped to chromosome
456	Xyl ⁻	22/82/nd	P	
457	Xyl ⁻	22/82/nd	P	mapped to chromosome
458	Mal ⁻	22/82/nd	P	mapped to chromosome
459				excluded
460	Xyl ⁻	22/88/nd	P	mapped to chromosome
461	Mal ⁻	22/91/nd	P	mapped to chromosome
462	Mal ⁻	22/91/nd	P	mapped to chromosome
463				excluded
464	Mal ⁻ Xyl ⁻	22/93/nd	P	mapped as separate mutations on chromosome
465	Mal ⁻	22/95/nd	P	
466	Xyl ⁻	22/95/nd	P	mapped to chromosome
467	Xyl ⁻	22/96/nd	P	mapped to chromosome
468	Xyl ⁻	22/97/nd	P	mapped to chromosome
469	Mal ⁻	22/98/nd	P	
470	Mal ⁻	22/98/nd	P	mutator mapped to <i>mutS</i>
471	Xyl ⁻	22/100/nd	P	mapped to chromosome
472	Xyl ⁻	22/100/nd	P	
473	Mal ⁻	22/103/nd	P	
474	Mal ⁻	22/105/nd	P	
475				excluded
476				excluded
477	Mal ⁻	22/106/nd	P	mapped to chromosome

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
478				excluded
479	Xyl ⁻	22/111/nd	P	
480	Mal ⁻	22/111/nd	P	mapped to chromosome
481	Xyl ⁻	22/13/nd	P	
482	Mal ⁻	22/6/nd	P	
483				excluded
484	Mal ⁻	22/22/nd	P	
485	Mal ⁻	22/20/nd	P	
486	Mal ⁻	22/36/nd	P	mapped to chromosome
487	Xyl ⁻	22/41/nd	P	
488	Xyl ⁻	22/43/nd	P	
489				excluded
490				excluded
491	Fruc ⁻	22/26/nd	P	
492				excluded
493				excluded
494				excluded
495				excluded
496				excluded
497				excluded
498				excluded
499				excluded
500	5FUR	22/1/3	P	mapped to <i>upp</i>
501	5FUR	22/1/5	P	mapped to <i>upp</i>
502	5FCR	22/1/7	P	mapped to <i>codAB</i>
503	5FUR	22/1/nd	P	mapped to <i>upp</i>
504	5FCR	22/1/nd	P	
505	5FCR	22/2/6	P	
506	5FUR	22/2/nd	P	mapped to <i>upp</i>
507	5FCR	22/2/nd	P	
508	5FCR	22/2/nd	P	
509	5FUR	22/2/nd	P	mapped to <i>upp</i>
510	5FCR	22/3/nd	P	mapped to <i>codAB</i>
511	5FCR	22/5/6	P	
512	5FUR	22/5/4	P	mapped to <i>upp</i>

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
513	5FUR	22/6/6	P	mapped to <i>upp</i> ; mutator mapped to <i>mutL</i>
514	5FUR	22/7/7	P	mapped to <i>upp</i>
515	5FCR	22/7/3	P	mapped to <i>codAB</i>
516	5FCR	22/7/nd	P	mapped to <i>codAB</i>
517	5FUR	22/7/nd	P	mapped to <i>upp</i>
518	5FCR	22/7/nd	P	mapped to <i>codAB</i>
519	5FCR	22/7/nd	P	mapped to <i>codAB</i>
520	5FCR	22/7/nd	P	mapped to <i>codAB</i>
521	5FUR	22/8/nd	P	mapped to <i>upp</i>
522	5FCR	22/8/nd	P	mapped to <i>codAB</i>
523	5FCR	22/9/7	P	mapped to <i>codAB</i>
524	5FUR	22/9/nd	P	mapped to <i>upp</i>
525	5FCR	22/9/nd	P	mapped to <i>codAB</i>
526	5FUR	22/11/6	P	
527	5FCR	22/12/nd	P	mapped to <i>codAB</i>
528	5FUR Xyl ⁻ Mal ⁻	22/13/nd	P	mutator not mapped
529	5FUR	22/13/nd	P	
530	5FUR	22/13/nd	P	
531	5FUR	22/14/6	P	
532	5FCR	22/14/nd	P	
533	5FCR	22/14/nd	P	
534	5FCR	22/14/nd	P	
535	5FUR	22/15/nd	P	
536	5FCR	22/16/7	P	
537	5FUR	22/16/nd	P	
538	5FUR	22/17/6	P	mapped to <i>upp</i>
539	5FCR	22/17/nd	P	
540	5FCR	22/19/nd	P	
541	5FUR	22/20/nd	P	
542	5FUR	22/20/nd	P	
543	5FCR	22/21/7	P	
544	5FUR	22/21/nd	P	
545	5FUR	22/21/nd	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
546	5FUR	22/21/nd	P	
547	5FUR	22/22/nd	P	
548	5FUR	22/22/nd	P	
549	5FUR	22/22/nd	P	
550	5FUR	22/23/nd	P	
551	5FUR	22/25/6	P	
552	5FUR	22/25/6	P	
553	5FUR	22/25/nd	P	
554	5FUR	22/26/nd	P	
555	5FUR	22/26/nd	P	
556	5FUR Mal ⁻	22/27/nd	P	5FUR mapped to <i>upp</i> ; Mal ⁻ mapped to chromosome
557	5FUR	22/28/4	P	
558	5FCR	22/28/nd	P	
559	5FUR	22/28/nd	P	
560	5FUR	22/30/nd	P	
561	5FUR	22/30/nd	P	
562	5FCR	22/32/7	P	
563	5FCR	22/32/nd	P	
564	5FUR	22/32/nd	P	
565	5FCR	22/32/nd	P	
566	5FUR	22/33/nd	P	
567	5FCR	22/33/nd	P	
568	5FUR	22/34/7	P	
569	5FCR	22/34/nd	P	
570	5FUR	22/34/nd	P	
571	5FUR	22/35/5	P	
572	5FCR	22/35/nd	P	
573	5FUR	22/35/nd	P	
574	5FUR	22/35/nd	P	
575	5FUR	22/37/7	P	
576	5FCR	22/37/nd	P	
577	5FUR	22/37/nd	P	
578	5FUR	22/38/7	P	mapped to <i>upp</i>
579	5FUR	22/38/7	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
580	5FCR	22/38/nd	P	mapped to <i>codAB</i>
581	5FUR	22/38/nd	P	
582	5FUR	22/39/nd	P	
583	5FUR	22/39/nd	P	
584	5FUR	22/40/nd	P	
585	5FUR	22/40/nd	P	
586	5FUR	22/42/nd	P	
587	5FCR	22/43/nd	P	
588	5FUR	22/43/nd	P	
589	5FUR	22/43/nd	P	
590	5FUR	22/43/nd	P	
591	5FUR	22/44/nd	P	
592	5FCR	22/44/nd	P	
593	5FCR	22/44/nd	P	
594	5FCR	22/44/nd	P	
595	5FCR	22/44/nd	P	
596	5FCR	22/46/nd	P	
597	5FCR	22/46/nd	P	
598	5FCR	22/48/nd	P	
599	5FCR	22/50/7	P	
600	5FUR	22/50/nd	P	
601	5FCR	22/51/7	P	
602	5FCR	22/51/nd	P	
603	5FCR	22/51/nd	P	
604	5FUR	22/51/nd	P	
605	5FUR	22/52/7	P	
606	5FUR	22/52/nd	P	
607	5FCR	22/53/nd	P	mapped to <i>codAB</i>
608	5FCR	22/53/nd	P	
609	5FCR	22/55/6	P	
610	5FUR	22/55/6	P	
611	5FCR	22/55/6	P	
612	5FCR	22/55/7	P	
613	5FCR	22/55/nd	P	
614	5FUR	22/55/7	P	
615	5FCR	22/55/7	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
616	5FCR	22/55/nd	P	
617	5FCR	22/55/nd	P	
618	5FCR	22/55/nd	P	
619	5FCR	22/55/nd	P	
620	5FCR	22/56/nd	P	
621	5FCR	22/59/nd	P	
622	5FCR	22/61/nd	P	
623	5FCR	22/62/nd	P	
624	5FUR	22/63/7	P	
625	5FCR	22/63/7	P	
626	5FCR	22/63/5	P	mapped to <i>codAB</i>
627	5FCR Mal ⁻	22/63/nd	P	5FCR mapped to <i>codAB</i> ; Mal ⁻ mapped to chromosome
628	5FCR	22/63/nd	P	
629	5FUR	22/64/4	P	
630	5FCR	22/64/nd	P	
631	5FUR	22/64/nd	P	
632	5FUR	22/66/3	P	
633	5FCR	22/66/nd	P	
634	5FCR	22/67/6	P	
635	5FCR	22/68/nd	P	
636	5FUR	22/68/nd	P	
637	5FUR	22/69/7	P	
638	5FCR	22/69/nd	P	
639	5FCR	22/70/nd	P	
640	5FCR	22/70/nd	P	
641	5FUR	22/71/5	P	
642	5FCR	22/73/nd	P	mapped to <i>codAB</i>
643	5FUR	22/74/nd	P	
644	5FUR	22/74/nd	P	
645	5FCR	22/74/nd	P	
646	5FUR	22/75/nd	P	mapped to <i>upp</i>
647	5FCR	22/75/nd	P	
648	5FUR	22/75/nd	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
649	5FCR	22/75/nd	P	
650	5FUR	22/75/nd	P	
651	5FCR	22/76/7	P	
652	5FUR	22/77/5	P	
653	5FUR	22/77/nd	P	
654	5FCR	22/78/nd	P	
655	5FCR	22/79/6	P	
656	5FUR	22/79/nd	P	
657	5FUR	22/80/4	P	
658	5FCR	22/80/3	P	
659	5FCR	22/80/nd	P	
660	5FCR	22/80/nd	P	
661	5FUR	22/80/nd	P	
662	5FUR	22/80/nd	P	
663	5FCR	22/80/nd	P	
664	5FCR	22/80/nd	P	
665	5FUR	22/81/nd	P	
666	5FCR	22/81/nd	P	
667	5FCR	22/82/6	P	
668	5FUR	22/82/nd	P	
669	5FCR	22/83/7	P	mapped to <i>codAB</i>
670	5FCR	22/83/nd	P	
671	5FCR	22/83/nd	P	mapped to <i>codAB</i>
672	5FCR	22/83/nd	P	mapped to <i>codAB</i>
673	5FUR	22/83/nd	P	
674	5FUR	22/83/nd	P	
675	5FUR	22/83/nd	P	
676	5FCR	22/85/nd	P	
677	5FUR	22/85/nd	P	
678	5FUR	22/85/nd	P	
679	5FCR	22/87/nd	P	mapped to <i>codAB</i>
680	5FCR	22/87/nd	P	
681	5FUR	22/87/nd	P	
682	5FCR	22/87/nd	P	
683	5FUR	22/87/nd	P	
684	5FUR	22/88/7	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
685	5FCR	22/88/nd	P	
686	5FCR	22/89/nd	P	
687	5FCR	22/89/nd	P	
688	5FCR	22/90/nd	P	
689	5FCR	22/91/nd	P	
690	5FCR	22/91/nd	P	
691	5FUR	22/92/nd	P	
692	5FCR	22/92/nd	P	
693	5FCR	22/93/nd	P	
694	5FCR	22/93/nd	P	
695	5FUR	22/94/nd	P	
696	5FCR	22/94/nd	P	
697	5FCR	22/94/nd	P	mapped to <i>codAB</i>
698	5FUR	22/97/nd	P	
699	5FUR	22/97/nd	P	
700	5FUR	22/97/nd	P	
701	5FUR	22/98/nd	P	
702	5FUR	22/98/nd	P	
703	5FUR	22/98/nd	P	mapped to <i>upp</i>
704	5FUR	22/99/nd	P	
705	5FUR	22/99/nd	P	
706	5FUR	22/99/nd	P	
707	5FUR	22/99/nd	P	
708	5FCR	22/100/nd	P	
709	5FUR	22/100/nd	P	
710	5FUR	22/101/nd	P	
711	5FUR	22/101/nd	P	
712	5FUR	22/102/nd	P	
713	5FCR	22/102/nd	P	mapped to <i>codAB</i>
714	5FUR	22/103/nd	P	
715	5FUR	22/103/nd	P	
716	5FCR	22/103/nd	P	mapped to <i>codAB</i>
717	5FUR	22/103/nd	P	
718	5FCR	22/103/nd	P	
719	5FUR	22/104/nd	P	
720	5FUR	22/104/nd	P	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
721	5FUR	22/104/nd	P	mapped to <i>codAB</i>
722	5FCR	22/104/nd	P	
723	5FUR	22/105/nd	P	
724	5FUR	22/105/nd	P	
725	5FCR	22/106/nd	P	
726	5FCR	22/111/nd	P	
727	5FCR	22/111/nd	P	
728	5FCR	22/111/nd	P	
729	5FCR	22/111/nd	P	
730	5FCR	22/111/nd	P	
731	5FCR	22/111/nd	P	stressed excluded excluded excluded excluded excluded excluded excluded
732	5FCR	22/112/nd	P	
733	5FCR	22/112/nd	P	
734	5FUR	22/85/nd	P	
735	5FCR	22/3/nd	P	
736	5FCR	22/3/nd	P	
737	5FCR	22/4/5	P	
738	5FCR	22/13/7	P	
739	5FCR	22/33/nd	P	
740	5FUR	22/80/3	P	
741	5FCR	22/88/6	P	stressed excluded excluded excluded excluded excluded excluded excluded
742	Lac ⁻ 5FCR	22/70/nd	P	
743				
744				
745				
746				
747				
748				
749				
750				
751†	5FCR	21/1/2	O	
752	5FCR	21/1/3	O	
753	5FCR	21/1/3	O	
754	5FCR	21/2/4	O	
755	5FCR	21/2/4	O	
756	5FCR	21/2/5	O	
757	5FCR	21/2/5	O	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
758	5FCR	21/2/5	O	
759	5FCR	21/2/4	O	
760	5FCR	21/2/3	O	
761†	5FCR	21/2/2	O	
762	5FCR	21/3/5	O	pure colony
763	5FUR	21/3/5	O	pure colony
764	5FUR	21/3/5	O	
765	5FUR	21/3/5	O	
766	5FCR	21/3/4	O	
767	5FCR	21/4/3	O	
768	5FCR	21/4/4	O	
769	5FCR	21/4/4	O	sectored colony
770	5FCR	21/4/4	O	
771	5FCR	21/4/4	O	
772	5FCR	21/4/3	O	
773	5FCR	21/4/4	O	
774	5FCR	21/4/5	O	
775	5FCR	21/4/5	O	
776	5FCR	21/4/5	O	
777	5FCR	21/4/4	O	
778†	5FCR	21/5/2	O	sectored colony
779	5FUR	21/5/3	O	
780	5FUR	21/6/4	O	
781	5FCR	21/6/4	O	
782	5FCR	21/6/5	O	
783	5FUR	21/7/4	O	
784	5FCR	21/7/4	O	
785	5FUR	21/7/4	O	sectored colony
786	5FCR	21/7/5	O	
787	5FCR	21/7/4	O	
788	5FUR	21/7/4	O	
789	5FCR	21/9/3	O	
790	5FCR	21/10/3	O	
791	5FCR	21/10/3	O	
792	5FCR	21/10/4	O	
793	5FCR	21/10/3	O	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
794	5FUR	21/11/3	O	
795	5FCR	21/11/3	O	
796	5FCR	21/11/3	O	
797	5FUR	21/11/4	O	
798†	5FCR	21/11/2	O	
799	5FCR	21/11/5	O	pure colony
800	5FUR	21/11/5	O	
801	5FCR	21/11/4	O	
802	5FCR	21/12/4	O	
803	5FCR	21/12/4	O	
804	5FCR	21/12/5	O	
805	5FCR	21/12/4	O	
806	5FCR	21/12/3	O	
807	5FCR	21/12/3	O	
808	5FCR	21/12/3	O	
809	5FCR	21/13/4	O	
810	5FCR	21/14/3	O	
811	5FCR	21/14/3	O	
812	5FCR	21/15/5	O	pure colony
813	5FUR	21/15/4	O	
814	5FCR	21/16/5	O	
815	5FCR	21/16/3	O	pure colony
816	5FCR	21/17/5	O	
817	5FCR	21/17/5	O	
818	5FCR	21/17/4	O	sectored colony
819	5FCR	21/17/3	O	
820	5FCR	21/17/3	O	pure colony
821	5FCR	21/17/3	O	pure colony
822	5FCR	21/17/4	O	
823	5FCR	21/17/3	O	
824	5FCR	21/17/3	O	
825	5FCR	21/17/3	O	
826	5FCR	21/18/4	O	
827	5FCR	21/18/4	O	
828	5FCR	21/18/4	O	
829	5FCR	21/18/5	O	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
830†	5FCR	21/18/2	O	
831	5FCR	21/18/5	O	pure colony
832	5FCR	21/18/5	O	
833	5FCR	21/18/4	O	
834	5FUR	21/18/3	O	
835	5FCR	21/18/4	O	
836	5FCR	21/18/4	O	
837	5FCR	21/18/5	O	
838	5FCR	21/19/4	O	
839	5FCR	21/20/4	O	
840	5FCR	21/20/3	O	
841	5FCR	21/20/5	O	
842	5FCR	21/20/3	O	pure colony
843	5FCR	21/20/5	O	
844	5FUR	21/20/4	O	
845	5FUR	21/20/5	O	pure colony
846				excluded
847				excluded
848				excluded
849				excluded
850				excluded
851	5FUR	22/1/3	O	
852	5FUR	22/1/5	O	
853	5FCR	22/1/7	O	
854	5FUR	22/1/nd	O	
855	5FCR	22/1/nd	O	pure colony
856	5FCR	22/2/6	O	
857	5FUR	22/2/nd	O	
858	5FCR	22/2/nd	O	pure colony
859	5FCR	22/2/nd	O	
860	5FUR	22/2/nd	O	
861	5FCR	22/3/nd	O	pure colony
862	5FCR	22/3/nd	O	pure colony
863	5FCR	22/3/nd	O	
864	5FCR	22/4/5	O	pure colony
865	5FCR	22/5/6	O	sectored colony

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
866	5FUR	22/5/4	O	
867	5FUR	22/6/6	O	pure colony
868	5FUR	22/7/7	O	
869	5FCR	22/7/3	O	
870	5FCR	22/7/nd	O	
871	5FUR	22/7/nd	O	
872	5FCR	22/7/nd	O	
873	5FCR	22/7/nd	O	
874	5FCR	22/7/nd	O	
875	5FUR	22/8/nd	O	
876	5FCR	22/8/nd	O	
877	5FCR	22/9/7	O	
878	5FUR	22/9/nd	O	pure colony
879	5FCR	22/9/nd	O	pure colony
880	5FUR	22/11/6	O	
881	5FCR	22/12/nd	O	pure colony
882	5FCR	22/13/7	O	
883	5FUR Xyl- Mal-	22/13/nd	O	
884	5FUR	22/13/nd	O	
885	5FUR	22/13/nd	O	
886	5FUR	22/14/6	O	sectored colony
887	5FCR	22/14/nd	O	
888	5FCR	22/14/nd	O	
889	5FCR	22/14/nd	O	
890	5FUR	22/15/nd	O	
891	5FCR	22/16/7	O	pure colony
892	5FUR	22/16/nd	O	
893	5FUR	22/17/6	O	
894	5FCR	22/17/nd	O	
895	5FCR	22/19/nd	O	
896	5FUR	22/20/nd	O	
897	5FUR	22/20/nd	O	
898	5FUR	22/20/nd	O	
899	5FCR	22/21/7	O	
900	5FUR	22/21/nd	O	
901	5FUR	22/21/nd	O	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
902	5FUR	22/21/nd	O	
903	5FUR	22/22/nd	O	
904	5FUR	22/22/nd	O	
905	5FUR	22/22/nd	O	
906	5FUR	22/23/nd	O	
907	5FUR	22/25/6	O	
908	5FUR	22/25/6	O	
909	5FUR	22/25/nd	O	
910	5FUR	22/26/nd	O	
911	5FUR	22/26/nd	O	
912	5FUR Mal ⁻	22/27/nd	O	
913	5FUR	22/28/4	O	
914	5FCR	22/28/nd	O	
915	5FUR	22/28/nd	O	
916	5FUR	22/30/nd	O	
917	5FUR	22/30/nd	O	
918	5FCR	22/32/7	O	
919	5FCR	22/32/nd	O	
920	5FUR	22/32/nd	O	
921	5FCR	22/32/nd	O	
922	5FUR	22/33/nd	O	
923	5FCR	22/33/nd	O	
924	5FCR	22/33/nd	O	
925	5FUR	22/34/7	O	
926	5FCR	22/34/nd	O	
927	5FUR	22/34/nd	O	
928	5FUR	22/35/5	O	
929	5FCR	22/35/nd	O	
930	5FUR	22/35/nd	O	
931	5FUR	22/35/nd	O	
932	5FUR	22/37/7	O	
933	5FCR	22/37/nd	O	
934	5FUR	22/37/nd	O	
935	5FUR	22/38/7	O	
936	5FUR	22/38/7	O	
937	5FCR	22/38/nd	O	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
938	5FUR	22/38/nd	O	
939	5FUR	22/39/nd	O	
940	5FUR	22/39/nd	O	
941	5FUR	22/40/nd	O	
942	5FUR	22/40/nd	O	
943	5FUR	22/42/nd	O	
944	5FCR	22/43/nd	O	
945	5FUR	22/43/nd	O	
946	5FUR	22/43/nd	O	
947	5FUR	22/43/nd	O	
948	5FUR	22/44/nd	O	
949	5FCR	22/44/nd	O	
950	5FCR	22/44/nd	O	
951	5FCR	22/44/nd	O	
952	5FCR	22/44/nd	O	
953	5FCR	22/46/nd	O	
954	5FCR	22/46/nd	O	
955	5FCR	22/48/nd	O	
956	5FCR	22/50/7	O	
957	5FUR	22/50/nd	O	
958	5FCR	22/51/7	O	
959	5FCR	22/51/nd	O	
960	5FCR	22/51/nd	O	
961	5FUR	22/51/nd	O	
962	5FUR	22/52/7	O	
963	5FUR	22/52/nd	O	
964	5FCR	22/53/nd	O	
965	5FCR	22/53/nd	O	
966	5FCR	22/55/6	O	
967	5FUR	22/55/6	O	
968	5FCR	22/55/6	O	
969	5FCR	22/55/7	O	
970	5FCR	22/55/nd	O	
971	5FUR	22/55/nd	O	
972	5FCR	22/55/nd	O	
973	5FCR	22/55/nd	O	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
974	5FCR	22/55/nd	O	
975	5FCR	22/55/nd	O	
976	5FCR	22/55/nd	O	
977	5FCR	22/56/nd	O	
978	5FCR	22/59/nd	O	
979	5FCR	22/61/nd	O	
980	5FCR	22/62/nd	O	
981	5FUR	22/63/7	O	
982	5FCR	22/63/7	O	
983	5FCR	22/63/5	O	
984	5FCR Mal-	22/63/nd	O	
985	5FCR	22/63/nd	O	
986	5FUR	22/64/4	O	
987	5FCR	22/64/nd	O	
988	5FUR	22/64/nd	O	
989	5FUR	22/66/3	O	
990	5FCR	22/66/nd	O	
991	5FCR	22/67/6	O	
992	5FCR	22/68/nd	O	
993	5FUR	22/68/nd	O	
994	5FUR	22/69/7	O	
995	5FCR	22/69/nd	O	
996	5FCR	22/70/nd	O	
997	5FCR	22/70/nd	O	
998	5FUR	22/71/5	O	
999	5FCR	22/73/nd	O	
1000	5FUR	22/74/nd	O	
1001	5FUR	22/74/nd	O	
1002	5FCR	22/74/nd	O	
1003	5FUR	22/75/nd	O	
1004	5FCR	22/75/nd	O	
1005	5FUR	22/75/nd	O	
1006	5FCR	22/75/nd	O	
1007	5FUR	22/75/nd	O	
1008	5FCR	22/76/7	O	
1009	5FUR	22/77/5	O	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
1010	5FUR	22/77/nd	O	
1011	5FCR	22/78/nd	O	
1012	5FCR	22/79/6	O	
1013	5FUR	22/79/nd	O	
1014	5FUR	22/80/4	O	
1015	5FUR	22/80/3	O	
1016	5FCR	22/80/3	O	
1017	5FCR	22/80/nd	O	
1018	5FCR	22/80/nd	O	
1019	5FUR	22/80/nd	O	
1020	5FUR	22/80/nd	O	
1021	5FCR	22/80/nd	O	
1022	5FCR	22/80/nd	O	
1023	5FUR	22/81/nd	O	
1024	5FCR	22/81/nd	O	
1025	5FCR	22/82/6	O	
1026	5FUR	22/82/nd	O	
1027	5FCR	22/83/7	O	
1028	5FCR	22/83/nd	O	
1029	5FCR	22/83/nd	O	
1030	5FCR	22/83/nd	O	
1031	5FUR	22/83/nd	O	
1032	5FUR	22/83/nd	O	
1033	5FUR	22/83/nd	O	
1034	5FCR	22/85/nd	O	
1035	5FUR	22/85/nd	O	
1036	5FUR	22/85/nd	O	
1037	5FCR	22/87/nd	O	
1038	5FCR	22/87/nd	O	
1039	5FUR	22/87/nd	O	
1040	5FCR	22/87/nd	O	
1041	5FUR	22/87/nd	O	
1042	5FUR	22/87/nd	O	
1043	5FCR	22/88/6	O	
1044	5FUR	22/88/7	O	
1045	5FCR	22/88/nd	O	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
1046	5FCR	22/89/nd	O	
1047	5FCR	22/89/nd	O	
1048	5FCR	22/90/nd	O	
1049	5FCR	22/91/nd	O	
1050	5FCR	22/91/nd	O	
1051	5FUR	22/92/nd	O	
1052	5FCR	22/92/nd	O	
1053	5FCR	22/93/nd	O	
1054	5FCR	22/93/nd	O	
1055	5FUR	22/94/nd	O	
1056	5FCR	22/94/nd	O	
1057	5FCR	22/94/nd	O	
1058	5FUR	22/97/nd	O	
1059	5FUR	22/97/nd	O	
1060	5FUR	22/97/nd	O	
1061	5FUR	22/98/nd	O	
1062	5FUR	22/98/nd	O	
1063	5FUR	22/98/nd	O	
1064	5FUR	22/99/nd	O	
1065	5FUR	22/99/nd	O	
1066	5FUR	22/99/nd	O	
1067	5FUR	22/99/nd	O	
1068	5FCR	22/100/nd	O	
1069	5FUR	22/100/nd	O	
1070	5FUR	22/101/nd	O	
1071	5FUR	22/101/nd	O	
1072	5FUR	22/102/nd	O	
1073	5FCR	22/102/nd	O	
1074	5FUR	22/103/nd	O	
1075	5FUR	22/103/nd	O	
1076	5FCR	22/103/nd	O	
1077	5FUR	22/103/nd	O	
1078	5FCR	22/103/nd	O	
1079	5FUR	22/104/nd	O	
1080	5FUR	22/104/nd	O	
1081	5FUR	22/104/nd	O	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
1082	5FCR	22/104/nd	O	
1083	5FUR	22/105/nd	O	
1084	5FUR	22/105/nd	O	
1085	5FCR	22/106/nd	O	
1086	5FCR	22/111/nd	O	
1087	5FCR	22/111/nd	O	
1088	5FCR	22/111/nd	O	
1089	5FCR	22/111/nd	O	
1090	5FCR	22/111/nd	O	
1091	5FCR	22/111/nd	O	
1092	5FCR	22/112/nd	O	
1093	5FCR	22/112/nd	O	
1094				excluded
1095				excluded
1096				excluded
1097				excluded
1098				excluded
1099				excluded
1100				excluded
1101	Mal ⁻	22/36/nd	O	
1102	Xyl ⁻	22/41/nd	O	
1103	Xyl ⁻	22/43/nd	O	
1104				excluded
1105				excluded
1106				excluded
1107				excluded
1108				excluded
1109	Xyl ⁻	22/51/nd	O	
1110				excluded
1111	Xyl ⁻	22/51/nd	O	
1112				excluded
1113	Mal ⁻	22/52/nd	O	
1114	Mal ⁻	22/53/nd	O	
1115	Mal ⁻	22/53/nd	O	
1116				excluded
1117				excluded
1118	Mal ⁻	22/56/nd	O	
1119				excluded

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
1120	Mal ⁻	22/57/nd	O	
1121	Mal ⁻	22/57/nd	O	
1122				excluded
1123	Xyl ⁻	22/58/nd	O	
1124	Mal ⁻	22/59/nd	O	
1125	Xyl ⁻	22/61/nd	O	
1126				excluded
1127				excluded
1128				excluded
1129	Xyl ⁻	22/62/nd	O	
1130				excluded
1131	Mal ⁻	22/63/nd	O	
1132				excluded
1133				excluded
1134				excluded
1135				excluded
1136				excluded
1137				excluded
1138	Xyl ⁻	22/70/nd	O	
1139	Mal ⁻	22/70/nd	O	
1140	Mal ⁻	22/71/nd	O	
1141	Mal ⁻	22/71/nd	O	
1142	Xyl ⁻	22/73/nd	O	
1143	Mal ⁻	22/73/nd	O	
1144				excluded
1145				excluded
1146	Mal ⁻ 5FCR	nd	O	
1147				excluded
1148				excluded
1149	Mal ⁻	22/79/nd	O	
1150				excluded
1151	Xyl ⁻	22/80/nd	O	
1152				excluded
1153	Xyl ⁻	nd		
1154	Xyl ⁻	22/80/nd	O	
1155				excluded
1156	Mal ⁻	22/81/nd	O	
1157				excluded

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
1158	Xyl ⁻	22/82/nd	O	
1159	Xyl ⁻	22/82/nd	O	
1160	Mal ⁻	22/82/nd	O	
1161				excluded
1162				excluded
1163				excluded
1164	Xyl ⁻	22/88/nd	O	
1165	Mal ⁻	22/91/nd	O	
1166	Mal ⁻	22/91/nd	O	
1167				excluded
1168				excluded
1169	Mal ⁻ Xyl ⁻	22/93/nd	O	
1170	Mal ⁻	nd	O	
1171	Mal ⁻	22/95/nd	O	sectored colony
1172	Xyl ⁻	22/95/nd	O	pure colony
1173	Xyl ⁻	22/96/nd	O	
1174	Xyl ⁻	22/97/nd	O	
1175	Mal ⁻	22/98/nd	O	pure colony
1176	Mal ⁻	22/98/nd	O	
1177	Xyl ⁻	22/100/nd	O	
1178	Xyl ⁻	22/100/nd	O	
1179	Mal ⁻	22/103/nd	O	
1180	Mal ⁻	22/105/nd	O	
1181				excluded
1182				excluded
1183	Mal ⁻	22/106/nd	O	
1184				excluded
1185	Xyl ⁻	22/111/nd	O	
1186	Mal ⁻	22/111/nd	O	
1187	Xyl ⁻	22/13/nd	O	
1188	Mal ⁻	22/6/nd	O	pure colony
1189				excluded
1190	Mal ⁻	22/22/nd	O	
1191				excluded
1192				excluded
1193	Fruc ⁻	22/26/nd	O	

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
1194	Mal ⁻ 5FCR	nd	P	Mal ⁻ mapped to chromosome; 5FCR mapped to <i>codAB</i>
1195	Xyl ⁻	nd	P	
1196	Mal ⁻	nd	P	
1197†	Ts	22/9/nd	O	minimal + rich
1198	Ts	22/9/nd	O	minimal
1199	Ts	22/9/nd	O	minimal
1200	Ts	22/22/nd	O	minimal
1201	Ts	22/34/nd	O	minimal
1202	Ts	22/73/nd	O	minimal
1203	Ts	22/74/nd	O	minimal
1204	Ts	22/76/nd	O	minimal
1205	Ts	22/89/nd	O	minimal
1206†	Ts	22/89/nd	O	minimal + rich
1207	Ts	22/94/nd	O	minimal
1208	Ts	22/82/nd	O	minimal
1209	Ts	22/9/nd	P	minimal
1210†	Ts	22/9/nd	P	minimal + rich
1211	Ts	22/9/nd	P	minimal
1212	Ts	22/22/nd	P	minimal
1213	Ts Mal ⁻	22/34/nd	P	minimal; Ts and Mal ⁻ mapped as separate mutations on chromosome
1214	Ts	22/73/nd	P	minimal
1215	Ts	22/74/nd	P	minimal
1216	Ts	22/76/nd	P	minimal
1217	Ts	22/89/nd	P	minimal
1218†	Ts	22/89/nd	P	minimal + rich
1219	Ts	22/94/nd	P	minimal
1220	Ts	22/82/nd	P	minimal
1221†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's
1222†	Lac ⁻ Tet ^R	na	P	growth-dependent

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
1223†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's
1224†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1T before run of 6G's
1225†	Lac ⁻ Tet ^R	na	P	growth-dependent
1226†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's
1227†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's
1228†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's
1229†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G after run of 6G's
1230†	Lac ⁻ Tet ^R	na	P	growth-dependent
1231†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's
1232†	Lac ⁻ Tet ^R	na	P	growth-dependent
1233†	Lac ⁻ Tet ^R	na	P	growth-dependent
1234†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's
1235†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
1236†	Lac ⁻ Tet ^R	na	P	growth-dependent
1237†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1T before run of 6G's
1238†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's
1239†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's
1240†	Lac ⁻ Tet ^R	na	P	growth-dependent
1241†	Lac ⁻ Tet ^R	na	P	growth-dependent
1242†	Lac ⁻ Tet ^R	na	P	growth-dependent
1243†	Lac ⁻ Tet ^R	na	P	growth-dependent; sequenced -1G in run of 6G's
1244	Lac ⁺ Tet ^S	na	P	pW17 transformed into SMR#970; used as control for media inducing mutations
1245	Lac ⁺ Tet ^S	na	P	pW17 transformed into SMR#973; used as control for media inducing mutations
1246	Lac ⁺ Tet ^S	na	P	pW17 transformed into SMR#974; used as control for media inducing mutations
1247	Lac ⁺ Tet ^S	na	P	pW17 transformed into SMR#976; used as control for media inducing mutations

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
1248	Lac ⁺ Tet ^S	na	P	pW17 transformed into SMR#978; used as control for media inducing mutations
1249	Lac ⁺ Tet ^S	na	P	pW17 transformed into SMR#994; used as control for media inducing mutations
1250	Lac ⁺ Tet ^S	na	P	pW17 transformed into SMR#1008; used as control for media inducing mutations
1251	Lac ⁺ Tet ^S	na	P	pW17 transformed into SMR#1017; used as control for media inducing mutations
1252	Lac ⁺ Tet ^S	na	P	pW17 transformed into SMR#1035; used as control for media inducing mutations
1253	Lac ⁺ Tet ^S	na	P	pW17 transformed into SMR#1043; used as control for media inducing mutations
1254†	Tet ^R pSL4	na	na	#77 mutator transformed with pSL4
1255†	Tet ^R pSL5	na	na	#77 mutator transformed with pSL5
1256†	Tet ^R pSL6	na	na	#77 mutator transformed with pSL6

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
1257†	Tet ^R pSL4	na	na	#84 mutator transformed with pSL4
1258†	Tet ^R pSL5	na	na	#84 mutator transformed with pSL5
1259†	Tet ^R pSL6	na	na	#84 mutator transformed with pSL6
1260†	Mal ⁻ pSL4	na	na	#424 mutator transformed with pSL4
1261†	Mal ⁻ pSL5	na	na	#424 mutator transformed with pSL5
1262†	Mal ⁻ pSL6	na	na	#424 mutator transformed with pSL6
1263†	Mal ⁻ pSL4	na	na	#470 mutator transformed with pSL4
1264†	Mal ⁻ pSL5	na	na	#470 mutator transformed with pSL5
1265†	Mal ⁻ pSL6	na	na	#470 mutator transformed with pSL6
1266†	5FUR ^R pSL4	na	na	#513 mutator transformed with pSL4
1267†	5FUR ^R pSL5	na	na	#513 mutator transformed with pSL5
1268†	5FUR ^R pSL6	na	na	#513 mutator transformed with pSL6
1269†	5FUR ^R Mal ⁻ Xyl ⁻ pSL4	na	na	#528 mutator transformed with pSL4

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
1270†	5FUR ^R Mal ⁻ Xyl ⁻ pSL5	na	na	#528 mutator transformed with pSL5
1271†	5FUR ^R Mal ⁻ Xyl ⁻ pSL6	na	na	#528 mutator transformed with pSL6
1272†	5FUR ^R Mal ⁻ Xyl ⁻ pGW1899	na	na	#528 mutator transformed with pGW1899
1273†	5FUR ^R Mal ⁻ Xyl ⁻ pGW1801	na	na	#528 mutator transformed with pGW1801
1274†	5FUR ^R Mal ⁻ Xyl ⁻ pdam118	na	na	#528 mutator transformed with pdam118
1275†	5FUR ^R Mal ⁻ Xyl ⁻ pTP166	na	na	#528 mutator transformed with pTP166
1276†	Tet ^S pRDK35	2/2/3	P	SMR# 1566
1277†	Tet ^S pRDK35	2/2/2	P	SMR# 1567
1278†	Tet ^S pRDK35	2/2/4	P	SMR# 1568
1279†	Tet ^S pRDK35	2/2/5	P	SMR# 1569
1280†	Tet ^S pW18	2/4/5	P	SMR# 1570
1281†	Tet ^S pW18	2/7/2	P	SMR# 1571
1282†	Tet ^S pW18	2/8/4	P	SMR# 1572
1283†	Tet ^S pW18	2/9/7	P	SMR# 1573
1284†	Tet ^R pRDK35	2/4/4	P	SMR# 1574
1285†	Tet ^R pRDK35	2/9/5	P	SMR# 1575
1286†	Tet ^R pW18	2/4/6	P	SMR# 1576
1287†	Tet ^R pW18	2/10/4	P	SMR# 1577
1288†	Lac ⁻ Tet ^R pRDK35	2/2/6	P	SMR# 1578
1289†	Lac ⁻ Tet ^R pRDK35	2/2/6	P	SMR# 1579
1290†	Lac ⁻ Tet ^R pW18	2/1/6	P	SMR# 1580
1291†	Lac ⁻ Tet ^R pW18	2/2/6	P	SMR# 1581
1292†	Tet ^R pRDK35	3/6/3	P	SMR# 1613

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/ day	O, R, or P	Notes
1293†	Lac ⁻ Tet ^R pRDK35	3/7/4	P	SMR# 1614
1294†	Lac ⁻ Tet ^R pRDK35	3/8/4	P	SMR# 1615
1295†	Lac ⁻ Tet ^R pW18	3/1/4	P	SMR# 1616
1296†	Lac ⁻ Tet ^R pW18	na	na	SMR# 1617 growth-dependent
1297†	Lac ⁻ Tet ^R pW18	na	na	SMR# 1645 growth-dependent
1298†	Lac ⁻ Tet ^R pRDK35	na	na	SMR# 1646 growth-dependent
1299†	Lac ⁻ Tet ^R pRDK35	na	na	SMR# 1647 growth-dependent
1300†	Tet ^R pRDK35	12/3/2	P	SMR# 1834
1301	Tet ^R pRDK35	12/3/3	P	SMR# 1835
1302†	Tet ^R pRDK35	12/3/nd	P	SMR# 1836
1303	Tet ^R pRDK35	12/3/5	P	SMR# 1837
1304†	Tet ^R pRDK35	12/4/2	P	SMR# 1838
1305†	Tet ^R pRDK35	12/4/2	P	SMR# 1839
1306	Tet ^R pRDK35	12/4/3	P	SMR# 1840
1307†	Tet ^R pRDK35	12/4/nd	P	SMR# 1841
1308†	Tet ^R pRDK35	12/4/nd	P	SMR# 1842
1309†	Tet ^R pRDK35	12/4/2	P	SMR# 1843
1310	Tet ^R pRDK35	12/4/3	P	SMR# 1844
1311	Tet ^R pRDK35	12/4/3	P	SMR# 1845
1312	Tet ^R pRDK35	12/4/6	P	SMR# 1846
1313†	Tet ^R pRDK35	12/4/2	P	SMR# 1847
1314	Tet ^R pRDK35	12/4/3	P	SMR# 1848
1315†	Tet ^R pRDK35	12/4/2	P	SMR# 1849
1316	Tet ^R pRDK35	12/4/5	P	SMR# 1850
1317†	Tet ^R pRDK35	12/5/2	P	SMR# 1851
1318†	Tet ^R pRDK35	12/5/nd	P	SMR# 1852
1319†	Tet ^R pRDK35	12/5/nd	P	SMR# 1853
1320†	Tet ^R pRDK35	12/5/nd	P	SMR# 1854
1321	Tet ^R pRDK35	12/5/6	P	SMR# 1855
1322	Tet ^R pRDK35	12/5/3	P	SMR# 1856

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
1323	Tet ^R pRDK35	12/5/3	P	SMR# 1857
1324†	Tet ^R pRDK35	12/5/2	P	SMR# 1858
1325†	Tet ^R pRDK35	12/5/2	P	SMR# 1859
1326†	Tet ^R pRDK35	12/6/2	P	SMR# 1860
1327†	Tet ^R pRDK35	12/6/2	P	SMR# 1861
1328†	Tet ^R pRDK35	12/6/2	P	SMR# 1862
1329†	Tet ^R pRDK35	12/6/2	P	SMR# 1863
1330†	FC40 recQ::Tn3 Tet ^R	12/3/2	P	SMR# 1864
1331† - 1374†	Lac ⁻ Tet ^R pW17	JN #1/nd/6	P	SMR# 1888 - 1931 stressed
1375†	Tet ^R pW18	JN #1/1/2	P	SMR# 1932
1376† - 1493†	Lac ⁺	13/na/gd	P	SMR# 2083 - 2200
* 1494 - 1611	Lac ⁻	14/nd/6	P	SMR# 2214 - 2331 stressed
1612	Lac ⁺	14/nd/a	P	SMR# 2332
1613 - 1708**	Lac ⁺	14/nd/a	P	SMR# 2342 - 2437
1709 - 1715	Lac ⁻	14/1/na	P	SMR# 2452 - 2458 unstressed
1716 - 1722	Lac ⁻	14/2/na	P	SMR# 2459 - 2465 unstressed
1723 - 1729	Lac ⁻	14/3/na	P	SMR# 2466 - 2472 unstressed
1730 - 1737	Lac ⁻	14/4/na	P	SMR# 2473 - 2480 unstressed
1738 - 1744	Lac ⁻	14/5/na	P	SMR# 2481 - 2487 unstressed
1745 - 1751	Lac ⁻	14/6/na	P	SMR# 2488 - 2493 unstressed
1752 - 1758	Lac ⁻	14/7/na	P	SMR# 2494 - 2500 unstressed

* Strain #1494 to 2211 were isolated as either Lac⁺ or Lac⁻ and then subsequently tested for a number of drug resistances, proline auxotrophy, fermentation defective phenotypes, 5FC^R, and 5FU^R. Not all are reported in chapter 2. See Joel Torkelson's data book for phenotypes of these strains.

** SMR#2389, 2393, and 2415 are 5FC^R and have been mapped to *codAB* (Unselected mutants 1660, 1664, & 1686 respectively).

Table B-1 continued...

Collection #	Phenotype	Expt#/culture/day	O, R, or P	Notes
1759 - 1764	Lac ⁻	14/8/na	P	SMR# 2501 - 2506 unstressed
1765 - 1770	Lac ⁻	14/9/na	P	SMR# 2507 - 2512 unstressed
1771 - 1776	Lac ⁻	14/10/na	P	SMR# 2513 - 2518 unstressed
1777 - 1783	Lac ⁻	14/11/na	P	SMR# 2519 - 2525 unstressed
1784 - 1790	Lac ⁻	14/12/na	P	SMR# 2526 - 2532 unstressed
1791 - 1797	Lac ⁻	14/13/na	P	SMR# 2533 - 2539 unstressed
1798 - 1803	Lac ⁻	14/14/na	P	SMR# 2540 - 2545 unstressed
1804 - 1810	Lac ⁻	14/15/na	P	SMR# 2546 - 2552 unstressed
1811 - 1817	Lac ⁻	14/16/na	P	SMR# 2553 - 2559 unstressed
1818 - 2111***	Lac ⁺	14/nd/4 or 5	P	SMR# 2606 - 2899
2112 - 2211	Lac ⁻	14/nd/5	P	SMR# 2900 - 2999 stressed

*** A number of these isolates are in strain background FC29 and not reported in chapter 2. See SMR collection for strain numbers.

Appendix C

***E. coli* K12 strains constructed or brought to the lab by Joel Torkelson**

Table C-1. A complete list of strains constructed or brought to the lab by Joel Torkelson.

SMR ¹ number	Strain name	Genotype	Construction information, source, reference and or notes
1877	JT1877	FC40 <i>recAo281</i> [pSL4]	#704 X pSL4 from #1790 miniprep
1878	JT1878	FC40 <i>recAo281</i> [pSL5]	#704 X pSL5 from #1713 miniprep
1879	JT1879	FC40 <i>recAo281</i> [pSL6]	#704 X pSL6 from #1747 miniprep
1880	JT1880	FC40 <i>recAo281</i> [pSL7]	#704 X pSL7 from #1791 miniprep
1881	JT1881	FC40 <i>recAo281</i> [pSL4]	#1301 X pSL4 from #1790 miniprep
1882	JT1882	FC40 <i>recAo281</i> [pSL5]	#1301 X pSL5 from #1713 miniprep
1883	JT1883	FC40 <i>recAo281</i> [pSL6]	#1301 X pSL6 from #1747 miniprep
1884	JT1884	FC40 <i>recAo281</i> [pSL7]	#1301 X pSL7 from #1791 miniprep
1885	JT1885	FC40 <i>zac3093::Tn10kan</i>	P1 #860 X #506
1886	JT1886	FC40 <i>recA0281</i> <i>zac3093::Tn10kan</i>	P1 #860 X #704
1887	JT1887	FC40 <i>recA0281</i> <i>zac3093::Tn10kan</i>	P1 #860 X #1301

¹ Susan M. Rosenberg

Table C-1 continued...

SMR number	Strain name	Genotype	Construction information, source, reference and or notes
1960	JT1960	FC40 <i>zac3093::Tn10kan</i> Lac ⁺	Lac ⁺ isolate #1 of #1885 obtained by plating on lactose-minimal kanamycin medium. Three additional, separate Lac ⁺ isolates from this selection are strains #1961, 1962, and 1963.
1961	JT1961	FC40 <i>zac3093::Tn10kan</i> Lac ⁺	Lac ⁺ isolate #2 of #1885
1962	JT1962	FC40 <i>zac3093::Tn10kan</i> Lac ⁺	Lac ⁺ isolate #3 of #1885
1963	JT1963	FC40 <i>zac3093::Tn10kan</i> Lac ⁺	Lac ⁺ isolate #4 of #1885
1964	JT1964	FC40 Lac ⁺ [pSL4]	Lac ⁺ isolate #1 of #1790 obtained by plating on lactose-minimal kanamycin medium. Three additional, separate Lac ⁺ isolates from this selection are strains #1965, 1966, and 1967.
1965	JT1965	FC40 Lac ⁺ [pSL4]	Lac ⁺ isolate #2 of #1790
1966	JT1966	FC40 Lac ⁺ [pSL4]	Lac ⁺ isolate #3 of #1790
1967	JT1967	FC40 Lac ⁺ [pSL4]	Lac ⁺ isolate #4 of #1790
1968	JT1968	FC40 Lac ⁺ [pSL7]	Lac ⁺ isolate #1 of #1791 obtained by plating on lactose-minimal kanamycin medium. Three additional, separate Lac ⁺ isolates from this selection are strains #1969, 1970, and 1971.
1969	JT1969	FC40 Lac ⁺ [pSL7]	Lac ⁺ isolate #2 of #1791

Table C-1 continued...

SMR number	Strain name	Genotype	Construction information, source, reference and or notes
1970	JT1970	FC40 Lac ⁺ [pSL7]	Lac ⁺ isolate #3 of #1791
1971	JT1971	FC40 Lac ⁺ [pSL7]	Lac ⁺ isolate #4 of #1791
1972	JT1972	FC40 Lac ⁺ [pSL6]	Lac ⁺ isolate #1 of #1747 obtained by plating on lactose-minimal kanamycin medium. Three additional, separate Lac ⁺ isolates from this selection are strains #1973, 1974, and 1975.
1973	JT1973	FC40 Lac ⁺ [pSL6]	Lac ⁺ isolate #2 of #1747
1974	JT1974	FC40 Lac ⁺ [pSL6]	Lac ⁺ isolate #3 of #1747
1975	JT1975	FC40 Lac ⁺ [pSL6]	Lac ⁺ isolate #4 of #1747
1976	JT1976	FC40 Lac ⁺ [pSL5]	Lac ⁺ isolate #1 of #1713 obtained by plating on lactose-minimal kanamycin medium. Three additional, separate Lac ⁺ isolates from this selection are strains #1977, 1978, and 1979.
1977	JT1977	FC40 Lac ⁺ [pSL5]	Lac ⁺ isolate #2 of #1713
1978	JT1978	FC40 Lac ⁺ [pSL5]	Lac ⁺ isolate #3 of #1713
1979	JT1979	FC40 Lac ⁺ [pSL5]	Lac ⁺ isolate #4 of #1713
1999	JT1999	FC40 <i>mutS::Tn10strep mutL218::Tn10</i> [pSL4]	#1787 X pSL4 from #1790 miniprep
2000	JT2000	FC40 <i>mutS::Tn10strep mutL218::Tn10</i> [pSL7]	#1787 X pSL7 from #1791 miniprep
3000	JT3000	FC40 <i>micA::Tn10kan</i>	P1 #312 X #506

Table C-1 continued...

SMR number	Strain name	Genotype	Construction information, source, reference and or notes
3001	BW271	W3110 <i>lac169</i> <i>phoA533</i> (Am S45) <i>IN(rrnD-rrnE)1 rpsL malt</i>	Strain recieved from Barry Wanner, Indiana University.
3002	BW723	FE103 <i>lac169</i> <i>phoA528::Tn5 IN(rrnD-rrnE)1 ilv-1 his-29</i> (Am) <i>trpA9605</i> (Am) <i>trpR tsx</i> <i>ara malt</i>	Strain recieved from Barry Wanner, Indiana University.
3003	BW3346	W3110 <i>lac169 proC::Tn5</i> <i>phoA533</i> (Am S45) <i>IN(rrnD-rrnE)1 rpsL trpR</i> <i>tna ilv bglR::IS</i>	Strain recieved from Barry Wanner, Indiana University.
3210	JWL163	<i>fhuA2 fhuA22 glnV44</i> (AS) λ^- <i>gatC49 gatA50 upp-33</i> <i>srlD50 rpsL104 malt1</i> (λ^R) <i>xylA7 mtlA2 metB1</i>	Strain recieved from CGSC. CGSC#6825. Reference: Lengeler 1975 <i>J. Bacteriol</i> 124:26.
3211	KMBL91	<i>thr-1 leuB6 fhuA2 lacY1</i> <i>codA1 glnV44</i> (AS) λ^- <i>pyrF101 rfbO1 thyA6</i> <i>uvrD502 thi-1 deoC1</i>	Strain recieved from CGSC. CGSC#5623. References: Seigel 1973 <i>J. Bacteriol</i> 113:145; Smirnov <i>et al.</i> 1971 <i>Mol. Gen.</i> <i>Genet.</i> 113:217
3281	JT3281	FC40 <i>upp-33</i>	P1 #3210 X #506
3290	R881	F ⁺ <i>lac-3350</i> λ^- <i>IN(rrnD-rrnE)1</i>	Strain recieved from CGSC. CGSC#6958.
3291	R874	F ⁺ <i>lac-3350</i> λ^- <i>bioF12</i> <i>IN(rrnD-rrnE)</i>	Strain recieved from CGSC. CGSC#6973. Reference: del Campillo-Campbell <i>et al.</i> <i>J.</i> <i>Bacteriol</i> 94:2065.
3300	JT3300	FC40 Lac ⁺ <i>upp-33</i>	P1 #3210 X #1134

Table C-1 continued...

SMR number	Strain name	Genotype	Construction information, source, reference and or notes
3382	JT3382	FC36 F ⁺	#3290 X #505 mated F ⁺ episome from R881 to FC36 and screened Rif ^R colonies for R17 ^S
3436	JT3436	FC36 Δ (<i>srl-recA</i>)306::Tn10 <i>zid3162</i> ::Tn10kan Strep ^R	P1 #624 X #3151
3445	SO113	F ⁻ <i>trp lacZ rpsL thi codA5</i>	Strain recieved from Jan Neuhard University Institute of Molecular Biology, Copenhagen Denmark
3446	SO122	F ⁻ <i>pyrF30 lacZ rpsL thi codA5</i>	Strain recieved from Jan Neuhard, University Institute of Molecular Biology, Copenhagen Denmark
3452	KUR1244	<i>pyrH(Ts) thi-1 thr-1 leuB6 proA2 lacY1 galK2 mtl-1 xyl15 ara-14 supE44 λ^-</i>	Strain recieved from Rod Kelln, University of Regina, Regina Saskatchewan
3471	SO463	F ⁻ <i>pyrF30 lacZ thi rpsL codB</i>	Strain recieved from Jan Neuhard, University Institute of Molecular Biology, Copenhagen Denmark
3666	JT3666	FC40 [pBR322]	#506 X pBR322 from #3650 miniprep
3677	JT3677	FC40 <i>mutU</i> ::Tn5 [pGW1801]	#621 X pGW1801 from #637 miniprep
3678	JT3678	FC40 <i>dam13</i> ::Tn9 [pdam118]	#843 X pdam118 from #1263 miniprep

Table C-1 continued...

SMR number	Strain name	Genotype	Construction information, source, reference and or notes
3679	JT3679	FC40 <i>dam13::Tn9</i> [pTP166]	#843 X pTP166 from #1264 miniprep
3680	JT3680	FC40 <i>mutH::Tn5</i> [pGW1899]	#623 X pGW1899 from #648 miniprep
3681	JT3681	FC40 [pGW1801]	#506 X pGW1801 from #637 miniprep
3689	CAG 18450	λ^- <i>zhg-50::Tn10 rph-1</i>	Recieved from CGSC. Carol Gross Tn10 mapping strain. CGSC#7450. Reference: Singer <i>et al.</i> 1989 <i>Microbiol. Rev.</i> 53:1.
3690	CAG 18475	λ^- <i>metC162::Tn10 rph-1</i>	Recieved from CGSC. Carol Gross Tn10 mapping strain. CGSC#7435. Reference: Singer <i>et al.</i> 1989 <i>Microbiol. Rev.</i> 53:1.
3691	CAG 18709	λ^- <i>zge-3074::Tn10 rph-1</i>	Recieved from CGSC. Carol Gross Tn10 mapping strain. CGSC#7430. Reference: Singer <i>et al.</i> 1989 <i>Microbiol. Rev.</i> 53:1.
3692	CAG 18427	λ^- <i>zje-2241::Tn10 rph-1</i>	Recieved from CGSC. Carol Gross Tn10 mapping strain. CGSC#7481. Reference: Singer <i>et al.</i> 1989 <i>Microbiol. Rev.</i> 53:1.

Appendix D

Calculation of hypermutable subpopulation¹

¹ I am grateful to P.J. Hastings for performing this calculation and allowing its presentation here.

Calculation of hypermutable subpopulation:

Given that:

$$p(x) = e^{-m} m^k / k!$$

From the numbers of Lac⁺ revertants and unselected mutants obtained in experiment 2 of Table 3-2, we can calculate that:

$$p(1) = 40,000/t; \quad p(2) = 300/t; \quad p(3) = 4/t; \quad e^{-m} \sim 1$$

then:

$$t = 40,000/m = 600/m^2 = 24/m^3 = 0.042/m^4$$

By approximation, $m = 0.02$ and hence $p(0) = 0.98$

Given that:

The median day of replica-plating is day 6, such that the Lac⁺ revertants arose over 4 days then $m = 0.02/4$ days or 0.005 mutations/day. Also $t = 2,000,000$ (this is the subpopulation from which the 40,000 Lac⁺ revertants arise).

We conclude that:

Mutation is random within the subpopulation.

The size of the subpopulation is as follows:

If we assume that at 6 days 100 Lac⁺ revertants arise per 10^8 cells plated, they must have come from $(100/40,000) \times 2,000,000 = 5,000$ cells. Therefore, the size of the subpopulation is $5,000/10^8 = 5 \times 10^{-5}$.